

10520 U.S. PTO  
07/07/99

Patent 244/101

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07/07/99

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Anticipated Classification of this application:  
Class 436 Subclass 514.900  
Prior application: 08/458,276  
Examiner: spiegel  
Art Unit: 1641

BOX PATENT APPLICATION  
Assistant Commissioner for Patents  
Washington, D. C. 20231

FILING UNDER 37 CFR § 1.53(B)

This is a request for filing for a

☐ continuation ☒ divisional ☐ continuation-in-part (CIP)

application under 37 CFR § 1.53(b) of pending prior application Serial No.  
08/633,248 filed on April 18, 1996, which names as inventors:

Kenneth F. Buechler and Paul H. McPherson

for: NOVEL METHODS FOR THE ASSAY OF TROPONIN I AND T AND  
COMPLEXES OF TROPONIN I AND T AND SELECTION OF ANTIBODIES  
FOR USE IN IMMUNOASSAYS

**I. COPY OF PRIOR APPLICATION AS FILED WHICH IS ATTACHED**

☒ I hereby verify that the attached papers are a true and complete copy of what  
is shown in my records to be the above-identified prior application, including the oath  
or declaration as originally filed. (37 CFR § 1.53)

95 Page(s) of Specification

SD-108679.1

CERTIFICATE OF MAILING  
(37 C.F.R. §1.10)

I hereby certify that this paper (along with any referred to as being attached or enclosed) is being deposited with the  
United States Postal Service on the date shown below with sufficient postage as 'Express Mail Post Office To Addressee'  
in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

EL088592668US  
Express Mail Label No.

July 7, 1999  
Date of Deposit

Diana Neuner  
Name of Person Mailing Paper

*Diana Neuner*  
Signature of Person Mailing Paper

32 Page(s) of Claims  
1 Page(s) of Abstract  
10 Sheet(s) of Drawings        formal X informal  
2 Page(s) of Declaration and Power of Attorney  
1 Page(s) of Small Entity Statement

- ☒ Pursuant to 37 CFR 1.63(d)(1), a newly executed oath or declaration is not required.  
☐ A newly executed oath or declaration is filed herewith.  
☒ I hereby state that the amendment referred to in the declaration filed to complete the prior application, in accordance with the requirements of 37 CFR § 1.53(b), did not introduce new matter therein.

## II. AMENDMENTS

- ☒ Cancel in this application original Claims 2-54 of the prior application before calculating the filing fee.  
☒ A Preliminary Amendment is enclosed.

## III. INFORMATION DISCLOSURE STATEMENT

- ☐ An Information Disclosure Statement, PTO 1449, and references are submitted herewith.

## IV. PETITION FOR SUSPENSION OF PROSECUTION FOR THE TIME TO FILE AN AMENDMENT

- ☐ There is provided herewith a PETITION FOR SUSPENSION OF PROSECUTION FOR THE TIME NECESSARY TO FILE AN AMENDMENT (NEW APPLICATION FILED CONCURRENTLY).

## V. FEE CALCULATION

BASIC FILING FEE:							\$760.00
Total Claims	80	-	20	=	60	x \$18.00	\$1,080.00
Independent Claims	16	-	3	=	13	x \$78.00	\$1,014.00
Multiple Dependent Claims	\$260	(if applicable)				<input checked="" type="checkbox"/>	\$260.00
Surcharge 37 CFR § 1.16(e)	\$130	(if applicable)				<input type="checkbox"/>	\$0.00
TOTAL OF ABOVE CALCULATIONS							\$3,114.00
Reduction by ½ for Filing by Small Entity. Note 37 CFR §§ 1.9, 1.27, 1.28. If applicable, Verified Statement must be attached.							\$1557.00

Misc. Filing Fees (Recordation of Assignment)	\$0.00
<b>TOTAL FEES SUBMITTED HEREWITH</b>	<b>\$1,557.00</b>

☐ The fee for extra claims is not being paid at this time.

## VI. SMALL ENTITY STATUS

A Verified Statement to establish small entity under 37 CFR §§ 1.9 and 1.27:

- ☐ is attached
- ☒ has been filed in the prior application and such status is still proper and desired. [37 CFR § 1.28(a)]

## VII. DRAWINGS

[NOTE: DO NOT CHECK THIS IF PRIOR CASE IS NOT TO BE ABANDONED.]

- ☐ Transfer the drawings from the prior application to this application and, subject to Item 16 below, abandon said prior application as of the filing date accorded to this application. A duplicate copy of this request is enclosed for filing in the prior application file.  
[May only be used if signed by (1) applicant, (2) assignee of record or (3) attorney or agent of record and before payment of issue fee. 37 CFR § 1.138.]
- ☐ Transfer the following sheet(s) of drawings from the prior application to this application.
- ☐ New drawings are enclosed ☐ formal ☐ informal

## VIII. PRIORITY - 35 USC § 119

- ☒ Priority of application Serial No. 08/633,248 filed on April 18, 1996, in USA, which is a continuation-in-part of U.S. Application Serial No. 08/423,582, filed April 18, 1995, which issued as U.S. Patent No. 5,795,725, is claimed under 35 USC § 119.
- ☐ The certified copy has been filed in prior U.S. application Serial No. \_\_\_\_\_ on \_\_\_\_\_.
- ☐ The certified copy will follow.

## IX. RELATE BACK - 35 USC § 120

- ☐ Amend the Specification by inserting before the first line the sentence:  
This is a continuation of co-pending application Serial No. \_\_\_\_\_ filed \_\_\_\_\_, which will issue as U.S. Patent No. on \_\_\_\_\_.

# **X. INVENTORSHIP STATEMENT**

☒ With respect to the prior co-pending U.S. application from which this application claims benefit under 35 USC § 120, the inventor(s) in this application is (are):

☒ the same

☐ less than those named in the prior application and it is requested that the following inventor(s) identified above for the prior application be deleted:

☒ The inventorship for all the claims in this application are:

☒ the same.

☐ not the same, and an explanation, including the ownership of the various claims at the time the last claimed invention was made, is submitted.

## **XI. ASSIGNMENT**

☒ The prior application is assigned of record to Biosite Diagnostics, Inc..

☐ An Assignment of the invention to \_\_\_\_\_ is attached.

## **XII. FEE PAYMENT BEING MADE AT THIS TIME**

☐ Not attached. No filing fee is submitted. [This and the surcharge required by 37 CFR § 1.16(e) can be paid subsequently.]

☒ Attached.

<input checked="" type="checkbox"/>	Filing fees.	<u>\$1,557</u>
<input type="checkbox"/>	Recording assignment. [\$40.00 37 CFR § 1.21(h)(1)]	—
	Petition fee for filing by other than all the inventors or person on behalf of the inventor where inventor refused to sign or cannot be reached.	
	[\$130.00; 37 CFR §§ 1.47 and 1.17(h)]	
<input type="checkbox"/>	Petition fee to Suspend Prosecution for the Time Necessary to File an Amendment (New Application Filed Concurrently.)	—
	[\$130.00; 37 CFR §§ 1.103 and 1.17(i)]	

- ☐ For processing an application with a specification in a non-English language. —  
 [\$130.00; 37 CFR §§ 1.52(d) and 1.17(k)]
- ☐ Processing and retention fee. —  
 [\$130.00; 37 CFR §§ 1.53(f) and 1.21(l)]
- Total Fees Enclosed**    \$1,557

### XIII. METHOD OF PAYMENT OF FEES

- ☒ Attached is a check in the amount of \$1,557.00.
- ☐ Charge Deposit Account No. **12-2475** in the amount of \_\_\_\_\_.

### XIV. AUTHORIZATION TO CHARGE ADDITIONAL FEES

The Commissioner is hereby authorized to charge the following additional fees by this paper and during the entire pendency of this application to Deposit Account No. **12-2475**:

- ☒ 37 CFR § 1.16(a) (filing fees).
- ☒ 37 CFR § 1.16(b) (presentation of extra claims).
- ☒ 37 CFR § 1.16(e) (surcharge for filing the basic filing fee and/or declaration on a date later than the filing date of the application).
- ☒ 37 CFR § 1.17 (application processing fees).
- ☒ 37 CFR § 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 CFR § 1.311(b)).

### XV. INSTRUCTIONS AS TO OVERPAYMENT

- ☒ Credit Deposit Account No. **12-2475**.
- ☐ Refund

### XVI. POWER OF ATTORNEY

- ☐ The power of attorney in the prior application is to \_\_\_\_\_.
- ☒ The power of attorney in the prior application is to the registered attorneys listed below and members of or associates in the law firm of **LYON & LYON LLP**, 633 West Fifth Street, 47<sup>th</sup> Floor, Los Angeles, California 90071, Registration No. 11,611, whose members are registered to practice in the U.S. Patent and Trademark office:

Roland N. Smoot, Reg. No. 18,718  
 Conrad R. Solum, Jr., Reg. No. 20,467  
 James W. Geriak, Reg. No. 20,233  
 Robert M. Taylor, Jr., Reg. No. 19,848  
 Samuel B. Stone, Reg. No. 19,297  
 Douglas E. Olson, Reg. No. 22,798  
 Robert E. Lyon, Reg. No. 24,171  
 Robert C. Weiss, Reg. No. 24,939  
 Richard E. Lyon, Jr., Reg. No. 26,300  
 John D. McConaghy, Reg. No. 26,733  
 William C. Steffin, Reg. No. 26,811  
 Coe A. Bloomberg, Reg. No. 26,605  
 J. Donald McCarthy, Reg. No. 25,119  
 John M. Benassi, Reg. No. 27,483  
 James J. Shalek, Reg. No. 29,749  
 Allan W. Jansen, Reg. No. 29,035  
 Robert W. Dickerson, Reg. No. 29,914  
 Roy L. Anderson, Reg. No. 30,240  
 David B. Murphy, Reg. No. 31,125

James C. Brooks, Reg. No. 29,898  
 Jeffrey M. Olson, Reg. No. 30,790  
 Steven D. Hemminger, Reg. No. 30,755  
 Jerrold B. Reilly, Reg. No. 32,293  
 Paul H. Meier, Reg. No. 32,274  
 John A. Rafter, Jr., Reg. No. 31,653  
 Kenneth H. Ohriner, Reg. No. 31, 646  
 Mary S. Consalvi, Reg. No. 32,212  
 Lois M. Kwasigroch, Reg. No. 35,579  
 Lawrence R. LaPorte, Reg. No. 38,948  
 Robert C. Laurensen, Reg. No. 34,206  
 Carol A. Schneider, Reg. No. 34,923  
 Hope E. Melville, Reg. No. 34,874  
 Michael J. Wise, Reg. No. 34,047  
 Richard J. Warburg, Reg. No. 32,327  
 Kurt T. Mulville, Reg. No. 37,194  
 Theodore S. Maceiko, Reg. No. 35,593  
 Bruce G. Chapman, Reg. No. 33,846  
 F. T. Alexandra Mahaney, Reg. No. 37,668

- ☐ The power appears in the original papers in the prior application.
- ☒ The power does not appear in the original papers, but was filed on July 10, 1996, in this application.
- ☐ A new power has been executed and is attached.
- ☐ Address all future communications to:

LYON & LYON LLP  
 633 West Fifth Street, 47<sup>th</sup> Floor  
 Los Angeles, California 90071-2066  
 (213) 489-1600

#### **XVII. MAINTENANCE OF CO-PENDENCY OF PRIOR APPLICATION**

- ☐ A petition, fee and response has been filed to extend the term in the pending **prior** application until \_\_\_\_\_. A copy of the petition for extension of time in the **prior** application is attached.

#### **XVIII. CONDITIONAL PETITIONS FOR EXTENSION OF TIME IN PRIOR APPLICATION**

- ☐ A conditional petition for extension of time is being filed in the pending **prior** application. A copy of the conditional petition for extension of time in the **prior** application is attached.

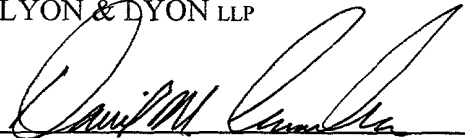
**XIX. ABANDONMENT OF PRIOR APPLICATION**

- ☐ Please abandon the prior application at a time while the prior application is pending or when the petition for extension of time or to revive in that application is granted and when this application is granted a filing date so as to make this application co-pending with said prior application. At the same time, please add the words "now abandoned" to the amendment of the specification set forth in Item 2 above.

Respectfully submitted,

LYON & DYON LLP

Dated: 07/07/99

By:   
For Richard J. Warburg,  
Daniel M. Chambers  
Registration No. 34,561

633 West Fifth Street, Suite 4700  
Los Angeles, California 90071-2066  
(213) 489-1600

Enclosures

Biosite Diagnostics, Inc.  
Name of Assignee

11030 Roselle Street, Suite D, San Diego, CA 92121  
Address of Assignee

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

**Kenneth F. Buechler and Paul H.  
McPherson**

Serial No.: Not Yet Assigned

Filed: Herewith

For: NOVEL METHODS FOR THE  
ASSAY OF TROPONIN I AND T  
AND COMPLEXES OF TROPONIN I  
AND T AND SELECTION OF  
ANTIBODIES FOR USE IN  
IMMUNOASSAYS

Group Art Unit: Not Yet Assigned

Examiner: Not Yet Assigned

TRANSMITTAL LETTERAssistant Commissioner for Patents  
Washington, D.C. 20231  
Box Patent Application

Sir:

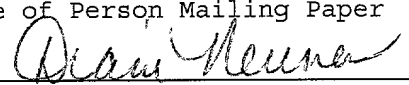
Transmitted herewith for filing in the above-referenced application are the

following:

SD-121583.1

CERTIFICATE OF MAILING  
(37 C.F.R. §1.10)I hereby certify that this paper (along with any referred to as being attached or enclosed) is being deposited with the United States Postal Service on the date shown below with sufficient postage as 'Express Mail Post Office To Addressee' in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.  
EL088592668US

Express Mail Label No.

July 7, 1999  
Date of DepositDiana Neuner  
Name of Person Mailing Paper  
Signature of Person Mailing Paper



- Patent Application Under 37 C.F.R. § 1.53(B), consisting of:
  - 95 Pages of Specification;
  - 32 Pages of Claims;
  - 10 Sheets of Drawings;
  - 2 Pages of Declaration and Power of Attorney; and
  - 1 Page of Small Entity Statement;
- Preliminary Amendment;
- Check in the amount of \$1557; and
- Return postcard.

The enclosed check includes \$1557 for the filing fee for the Patent Application Under 37 C.F.R. § 1.53(B). If the enclosed fee is incorrect, please charge or credit our Deposit Account No. 12-2475 for the appropriate amount.

Respectfully submitted,

LYON & LYON LLP

Dated

07/07/99

By



For Richard J. Warburg,  
Daniel M. Chambers  
Registration No. 34,561

First Interstate World Center  
633 West Fifth Street, 47th Floor  
Los Angeles, CA 90071-2066  
Telephone: (619) 552-8400  
Facsimile: (213) 955-0440



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

**Kenneth F. Buechler and Paul H.  
McPherson**

Serial No.: Not Yet Assigned

Filed: Herewith

For: NOVEL METHODS FOR THE  
ASSAY OF TROPONIN I AND T  
AND COMPLEXES OF TROPONIN I  
AND T AND SELECTION OF  
ANTIBODIES FOR USE IN  
IMMUNOASSAYS

Group Art Unit: Not Yet Assigned

Examiner: Not Yet Assigned

**PRELIMINARY AMENDMENT**

Assistant Commissioner for Patents  
Washington, D.C. 20231  
Box Patent Application

Please consider the following amendments and remarks in connection with the above-identified divisional application filed herewith.

**IN THE SPECIFICATION**

On page 1, lines 3-4, please replace "This application is a continuation-in-part of App. Ser. No. 08/423,582 filed April 18, 1995 from which priority is claimed" with -- This  
SD-108700.1

**CERTIFICATE OF MAILING**  
(37 C.F.R. §1.10)

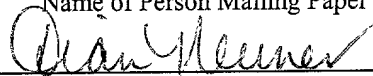
I hereby certify that this paper (along with any referred to as being attached or enclosed) is being deposited with the United States Postal Service on the date shown below with sufficient postage as 'Express Mail Post Office To Addressee' in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

EL088592668US  
Express Mail Label No.

July 7, 1999  
Date of Deposit

Diana Neuner

Name of Person Mailing Paper



Signature of Person Mailing Paper

application is a divisional of U.S. Application Serial No. 08/633,248 filed on April 18, 1996 (pending), which is a continuation-in-part of U.S. Application Serial No. 08/423,582 filed on April 18, 1995, which issued as U.S. Patent No. 5,795,725, each of which is hereby incorporated by reference, and from each of which priority is claimed --.

### IN THE CLAIMS

Please cancel claims 2-54 without prejudice to future prosecution. Please add the following claims.

55. An assay for determining the presence or amount of complexes of a cardiac specific isoform of troponin in a patient sample, said assay comprising:

performing an immunoassay with an antibody which specifically binds said cardiac specific isoform of troponin in a complex comprising at least one other troponin component selected from the group consisting of troponin I, troponin C, and troponin T; and

detecting a signal from said immunoassay resulting from said antibody binding said complexes, wherein said signal is at least a factor of two larger than a minimum signal resulting from said antibody binding to an equal number of free troponin components and/or troponin complexes which do not comprise said cardiac specific isoform of troponin, and wherein said signal is related to the presence or amount of said complexes in said sample.

56. An assay according to claim 55, wherein said patient sample is selected from the group consisting of a blood sample, a serum sample, and a plasma sample.

57. An assay according to claim 55, wherein said signal is at least a factor of five greater than said minimum signal.

58. An assay according to claim 55, wherein the immunoassay comprises a sandwich immunoassay.

59. An assay according to claim 55, wherein said antibody is conjugated to a signal development element.

60. An assay according to claim 55, wherein said immunoassay comprises providing an antibody immobilized on a solid phase, the immobilized antibody binding said cardiac specific isoform of troponin.

61. An assay according to claim 55, wherein said antibody is conjugated to a signal development element, and wherein said immunoassay comprises providing an antibody immobilized on a solid phase, the immobilized antibody binding said cardiac specific isoform of troponin.

62. An assay according to claim 55, wherein said immunoassay provides a quantitative assay for said cardiac specific isoform of troponin.

63. An assay according to claim 55, wherein said cardiac specific isoform of troponin is cardiac specific troponin I.

64. An assay according to claim 55, wherein said cardiac specific isoform of troponin is cardiac specific troponin T.

65. An assay according to claim 55, wherein said assay provides a method for diagnosing a myocardial infarction.

66. An assay according to claim 55, wherein said cardiac specific isoform of troponin is cardiac specific troponin I, and said antibody specifically binds complexes comprising an oxidized form of cardiac specific troponin I.

67. An assay according to claim 55, wherein said cardiac specific isoform of troponin is cardiac specific troponin I, and said antibody specifically binds complexes comprising a reduced form of cardiac specific troponin I.

68. An assay according to claim 55, wherein said cardiac specific isoform of troponin is cardiac specific troponin I, and said antibody specifically binds complexes comprising

oxidized and reduced forms of cardiac specific troponin I.

69. An immunoassay for determining the presence or amount of complexes of a cardiac specific isoform of troponin in a patient sample, said immunoassay comprising:

a) contacting a sample with an antibody conjugate comprising a signal development element and an antibody which specifically binds said cardiac specific isoform of troponin in a complex comprising at least one other troponin component selected from the group consisting of troponin I, troponin C, and troponin T; or

contacting a sample with an antibody conjugate comprising a signal development element and an antibody which specifically binds said cardiac specific isoform of troponin in a binary complex comprising one other troponin component selected from the group consisting of troponin I, troponin C, and troponin T; and, contacting the sample with an antibody conjugate comprising a signal development element and an antibody which specifically binds said cardiac specific isoform of troponin in a ternary complex comprising two other troponin components selected from the group consisting of troponin I, troponin C, and troponin T;

b) contacting the mixture of step (a) with a solid phase comprising at least one capture antibody which specifically binds said complexes which are bound to antibody conjugate or which specifically binds said complexes which are bound to antibody conjugate following binding to the capture antibody; and

c) generating a detectable signal from the antibody conjugate following binding to the solid phase of cardiac specific troponin complexes which are or become bound to antibody conjugate, wherein said detectable signal is at least a factor of two larger than a minimum signal resulting from said antibody conjugate(s) binding to an equal number of free troponin components and/or troponin complexes which do not comprise said cardiac specific isoform of troponin, and wherein the detectable signal is related to the presence or amount of said complexes in said sample.

70. An immunoassay according to claim 69, wherein the patient sample is selected from the group consisting of a blood sample, a serum sample, and a plasma sample.

71. An immunoassay according to claim 69, wherein said detectable signal is at least a factor of five greater than said minimum signal.

72. An immunoassay for determining the presence or amount of complexes of any cardiac specific isoform of troponin in a patient sample, said immunoassay comprising:

a) contacting a sample with an antibody conjugate comprising a signal development element and an antibody which specifically binds a cardiac specific isoform of troponin in a complex comprising at least one other troponin component selected from the group consisting of troponin I, troponin C and troponin T; or

contacting a sample with an antibody conjugate comprising a signal development element and an antibody which specifically binds a cardiac specific isoform of troponin in a binary complex comprising one other troponin component selected from the group consisting of troponin I, troponin C and troponin T; and contacting the sample with an antibody conjugate comprising a signal development element and an antibody which specifically binds said cardiac specific isoform of troponin in a ternary complex comprising two other troponin components selected from the group consisting of troponin I, troponin C and troponin T;

b) contacting the mixture of step (a) with a solid phase comprising at least one capture antibody which specifically binds said complexes which are bound to antibody conjugate or which specifically binds said complexes which are bound to antibody conjugate following binding to the capture antibody; and

c) generating a detectable signal from the antibody conjugate following binding to the solid phase of cardiac specific troponin complexes which are or become bound to antibody conjugate, wherein said detectable signal is at least a factor of two larger than a minimum signal resulting from said antibody conjugate(s) binding to an equal number of free troponin components and/or troponin complexes which do not comprise said cardiac specific isoform of troponin, and wherein the detectable signal is related to the presence or amount of said complexes in said sample.

73. An immunoassay according to claim 72, wherein the patient sample is selected

from the group consisting of a blood sample, a serum sample, and a plasma sample.

74. An immunoassay according to claim 72, wherein said detectable signal is approximately equal for equal amounts of all cardiac specific isoforms of troponin.

75. An immunoassay according to claim 72, wherein said detectable signal is within a factor of 0.2 for equal amounts of all cardiac specific isoforms of troponin.

76. An immunoassay according to claim 72, wherein said detectable signal is within a factor of 2 for equal amounts of all cardiac specific isoforms of troponin.

77. An immunoassay according to claim 72, wherein said detectable signal is at least a factor of five greater than said minimum signal.

78. An assay for determining the presence or amount of complexes of any cardiac specific isoform troponin in a patient sample, said assay comprising:

performing an immunoassay with an antibody which specifically binds a cardiac specific isoform of troponin in a complex comprising at least one other troponin component selected from the group consisting of troponin I, troponin C and troponin T; and

detecting a signal from said immunoassay resulting from said antibody binding said complexes, wherein said signal is at least a factor of two larger than a minimum signal resulting from said antibody binding to an equal number of free troponin components and/or troponin complexes which do not comprise said cardiac specific isoform of troponin, and wherein said signal is related to the presence or amount of said complexes in said sample.

79. An assay according to claim 78, wherein said patient sample is selected from the group consisting of a blood sample, a serum sample, and a plasma sample.

80. An assay according to claim 78, wherein said signal is approximately equal for equal amounts of all cardiac specific isoforms of troponin.

81. An assay according to claim 78, wherein said detectable signal is within a factor of 0.2 for equal amounts of all cardiac specific isoforms of troponin.



82. An assay according to claim 78, wherein said signal is within a factor of 2 for equal amounts of all cardiac specific isoforms of troponin.

83. An assay according to claim 78, wherein said detectable signal is at least a factor of five greater than said minimum signal.

84. An assay according to either one of claims 55 or 78, wherein said antibody comprises a plurality of antibodies selected from the group consisting of recombinant and monoclonal antibodies.

85. An assay for determining the presence or amount of a free and complexed cardiac specific isoform of troponin in a patient sample, said assay comprising:

performing an immunoassay with an antibody which specifically binds said free cardiac specific isoform of troponin, and which specifically binds said cardiac specific isoform of troponin in a complex comprising at least one other troponin component selected from the group consisting of troponin I, troponin C and troponin T; and

detecting a signal from said immunoassay resulting from said antibody binding said free and complexed cardiac specific isoform of troponin, wherein said signal is at least a factor of two larger than a minimum signal resulting from said antibody binding to an equal number of free troponin components which are not said cardiac specific isoform of troponin and/or troponin complexes which do not comprise said cardiac specific isoform of troponin, and wherein said signal is related to the presence or amount of said free and complexed cardiac specific isoform of troponin in said sample.

86. An assay according to claim 85, wherein said patient sample is selected from the group consisting of a blood sample, a serum sample, and a plasma sample.

87. An assay according to claim 85, wherein said detectable signal is at least a factor of five greater than said minimum signal.

88. An assay for determining the presence or amount of a free and complexed cardiac specific isoform of troponin in a patient sample, said assay comprising:

performing an immunoassay with an antibody which specifically binds to free cardiac specific troponin I and/or cardiac specific troponin T, and which binds to cardiac specific troponin I and/or cardiac specific troponin T in a complex comprising at least one other troponin component selected from the group consisting of troponin I, troponin C and troponin T; and

detecting a signal from said immunoassay resulting from said antibody binding said free and complexed cardiac specific isoform of troponin, wherein said signal is at least a factor of two larger than a minimum signal resulting from said antibody binding to an equal number of free troponin components which are not said cardiac specific isoform of troponin and/or troponin complexes which do not comprise said cardiac specific isoform of troponin, and wherein said signal is related to the presence or amount of said free and complexed cardiac specific isoform of troponin in said sample.

89. An assay according to claim 88, wherein said patient sample is selected from the group consisting of a blood sample, a serum sample, and a plasma sample.

90. An assay according to claim 88, wherein said signal is at least a factor of five greater than said minimum signal.

91. An assay for determining the presence or amount of free and complexed cardiac specific troponin I in a patient sample, said assay comprising:

performing an immunoassay with an antibody which specifically binds to free cardiac specific troponin I, and which binds to cardiac specific troponin I in a complex comprising at least one other troponin component selected from the group consisting of troponin C and troponin T; and

detecting a signal from said immunoassay resulting from said antibody binding said free and complexed cardiac specific troponin I, wherein said signal is at least a factor of two larger than a minimum signal resulting from said antibody binding to an equal number of free troponin components which are not said cardiac specific troponin I and/or troponin complexes which do not comprise said cardiac specific troponin I, and wherein said detectable signal is related to the presence or amount of said free and complexed cardiac specific troponin I in said sample.

92. An assay according to claim 91, wherein said patient sample is selected from the group consisting of a blood sample, a serum sample, and a plasma sample.

93. An assay according to claim 91, wherein said detectable signal is at least a factor of five greater than said minimum signal.

94. An assay for determining the presence or amount of free and complexed cardiac specific troponin T in a patient sample, said assay comprising:

performing an immunoassay with an antibody which specifically binds to free cardiac specific troponin T, and which binds to cardiac specific troponin T in a complex comprising at least one other troponin component selected from the group consisting of troponin I and troponin C; and

detecting a signal from said immunoassay resulting from said antibody binding said free and complexed cardiac specific troponin T, wherein said signal is at least a factor of two larger than a minimum signal resulting from said antibody binding to an equal number of free troponin components which are not said cardiac specific troponin T and/or troponin complexes which do not comprise said cardiac specific troponin T, and wherein said signal is related to the presence or amount of said free and complexed cardiac specific troponin T in said sample.

95. An assay according to claim 94, wherein said patient sample is selected from the group consisting of a blood sample, a serum sample, and a plasma sample.

96. An assay according to claim 94, wherein said detectable signal is at least a factor of five greater than said minimum signal.

97. An immunoassay for determining the presence or amount of complexes of any cardiac specific isoform of troponin in a patient sample, said immunoassay comprising:

a) contacting a sample with an antibody conjugate comprising a signal development element and an antibody which specifically binds to troponin complexes comprising at least two troponin components selected from the group consisting of troponin I, troponin C and troponin T; or

contacting a sample with an antibody conjugate comprising a signal development element and an antibody which specifically binds to troponin binary complexes comprising two troponin components selected from the group consisting of troponin I, troponin C and troponin T; and contacting the sample with an antibody conjugate comprising a signal development element and an antibody which specifically binds troponin ternary complexes comprising troponin I, troponin C and troponin T, and;

b) contacting the mixture of step (a) with a solid phase comprising at least one capture antibody which specifically binds said complexes comprising a cardiac specific isoform of troponin which are bound to antibody conjugate, or which specifically binds said complexes comprising said cardiac specific isoform of troponin which are bound to antibody conjugate after binding to capture antibody; and

c) generating a detectable signal from the antibody conjugate following binding to the solid phase of said complexes comprising said cardiac specific isoform of troponin which are or become bound to antibody conjugate, wherein the detectable signal is related to the presence or amount of said complexes comprising said cardiac specific isoform of troponin in said sample.

98. An assay according to claim 97, wherein said patient sample is selected from the group consisting of a blood sample, a serum sample, and a plasma sample.

99. An assay according to claim 97, wherein said detectable signal is approximately equal for equal amounts of all cardiac specific isoforms of troponin.

100. An assay according to claim 97, wherein said detectable signal is within a factor of 0.2 for equal amounts of all cardiac specific isoforms of troponin.

101. An assay according to claim 97, wherein said detectable signal is within a factor of 2 for equal amounts of all cardiac specific isoforms of troponin.

102. An assay for determining the presence or amount of all free and complexed cardiac specific isoform of troponin in a patient sample, said assay comprising:

performing an immunoassay with an antibody which specifically binds any free cardiac

specific isoform of troponin, and which specifically binds any cardiac specific isoform of troponin in a complex comprising at least one other troponin component selected from the group consisting of troponin I, troponin C and troponin T; and

detecting a signal from said immunoassay resulting from said antibody binding said free and complexed cardiac specific isoforms of troponin, wherein said signal is related to the presence or amount of all free and complexed cardiac specific isoform of troponin in said sample.

103. An assay according to claim 102, wherein said patient sample is selected from the group consisting of a blood sample, a serum sample, and a plasma sample.

104. An assay according to claim 102, wherein said signal is approximately equal for equal amounts of all cardiac specific isoforms of troponin.

105. An assay according to claim 102, wherein said signal is within a factor of 0.2 for equal amounts of all cardiac specific isoforms of troponin.

106. An assay according to claim 102, wherein said signal is within a factor of 2 for equal amounts of all cardiac specific isoforms of troponin.

107. An assay for determining the presence or amount of all complexed cardiac specific isoform of troponin in a patient sample, said assay comprising:

performing an immunoassay with an antibody which specifically binds any cardiac specific isoform of troponin in a complex comprising at least one other troponin component selected from the group consisting of troponin I, troponin C and troponin T; and

detecting a signal from said immunoassay resulting from said antibody binding said complexed cardiac specific isoforms of troponin, wherein said signal is related to the presence or amount of all complexed cardiac specific isoforms of troponin in said sample.

108. An assay according to claim 107, wherein said patient sample is selected from the group consisting of a blood sample, a serum sample, and a plasma sample.

109. An assay according to claim 107, wherein said signal is approximately equal for equal amounts of all cardiac specific isoforms of troponin.

110. An assay according to claim 107, wherein said signal is within a factor of 0.2 f for equal amounts of all cardiac specific isoforms of troponin.

111. An assay according to claim 107, wherein said signal is within a factor of 2 for equal amounts of all cardiac specific isoforms of troponin.

112. An assay according to either one of claims 93 or 107, wherein said immunoassay provides a quantitative assay for any complexed cardiac specific isoform of troponin.

113. An assay according to either one of claims 102 or 107, wherein said antibody comprises a plurality of antibodies selected from the group consisting of recombinant and monoclonal antibodies.

114. An assay for determining the presence or amount of a free and complexed cardiac specific isoform of troponin in a patient sample, said assay comprising:

performing an immunoassay with an antibody which specifically binds said free cardiac specific isoform of troponin, and which specifically binds said cardiac specific isoform of troponin in a complex comprising at least one other troponin component selected from the group consisting of troponin I, troponin C and troponin T; and

detecting a signal from said immunoassay resulting from said antibody binding said free and complexed cardiac specific isoform of troponin, wherein said signal is related to the presence or amount of said free and complexed cardiac specific isoform of troponin in said sample.

115. An assay according to claim 114, wherein said patient sample is selected from the group consisting of a blood sample, a serum sample, and a plasma sample.

116. An assay according to claim 114, wherein said signal is approximately equal for equal amounts of said free cardiac specific isoform of troponin and said complexed cardiac specific isoform of troponin.

117. An assay according to claim 114, wherein said signal is within a factor of 0.2 for equal amounts of said free cardiac specific isoform of troponin and said complexed cardiac specific isoform of troponin.

118. An assay according to claim 114, wherein said detectable signal is within a factor of 2 for equal amounts of said free cardiac specific isoform of troponin and said complexed cardiac specific isoform of troponin.

119. An assay for determining the presence or amount of a free and complexed cardiac specific isoform of troponin in a patient sample, said assay comprising:

performing an immunoassay with an antibody which specifically binds to free cardiac specific troponin I and/or cardiac specific troponin T, and which binds to cardiac specific troponin I and/or cardiac specific troponin T in a complex comprising at least one other troponin component selected from the group consisting of troponin I, troponin C and troponin T; and

detecting a signal from said immunoassay resulting from said antibody binding said free and complexed cardiac specific isoform of troponin, wherein said signal is related to the presence or amount of said free and complexed cardiac specific isoform of troponin in said sample.

120. An assay according to claim 119, wherein said patient sample is selected from the group consisting of a blood sample, a serum sample, and a plasma sample.

121. An assay according to claim 119, wherein said signal is approximately equal for equal amounts of said free cardiac specific isoform of troponin and said complexed cardiac specific isoform of troponin.

122. An assay according to claim 119, wherein said signal is within a factor of 0.2 for equal amounts of said free cardiac specific isoform of troponin and said complexed cardiac specific isoform of troponin.

123. An assay according to claim 119, wherein said signal is within a factor of 2 for equal amounts of said free cardiac specific isoform of troponin and said complexed cardiac specific isoform of troponin.

124. An assay for determining the presence or amount of free and complexed cardiac specific troponin I in a patient sample, said assay comprising:

performing an immunoassay with an antibody which specifically binds to free cardiac specific troponin I, and which binds to cardiac specific troponin I in a complex comprising at least one other troponin component selected from the group consisting of troponin C and troponin T; and

detecting a signal from said immunoassay resulting from said antibody binding said free and complexed cardiac specific troponin I, wherein said signal is related to the presence or amount of said free and complexed cardiac specific troponin I in said sample.

125. An assay according to claim 124, wherein said patient sample is selected from the group consisting of a blood sample, a serum sample, and a plasma sample.

126. An assay according to claim 124, wherein said signal is approximately equal for equal amounts of said free cardiac specific troponin I and said complexed cardiac specific troponin I.

127. An assay according to claim 124, wherein said signal is within a factor of 0.2 for equal amounts of said free cardiac specific troponin I and said complexed cardiac specific troponin I.

128. An assay according to claim 124, wherein said signal is within a factor of 2 for equal amounts of said free cardiac specific troponin I and said complexed cardiac specific troponin I.

129. An assay for determining the presence or amount of free and complexed cardiac specific troponin T in a patient sample, said assay comprising:

performing an immunoassay with an antibody which specifically binds to free cardiac specific troponin T, and which binds to cardiac specific troponin T in a complex comprising at least one other troponin component selected from the group consisting of troponin I and troponin C; and



detecting a signal from said immunoassay resulting from said antibody binding said free and complexed cardiac specific troponin T, wherein said signal is related to the presence or amount of said free and complexed cardiac specific troponin T in said sample.

130. An assay according to claim 129, wherein said patient sample is selected from the group consisting of a blood sample, a serum sample, and a plasma sample.

131. An assay according to claim 129, wherein said signal is approximately equal for equal amounts of said free cardiac specific troponin T and said complexed cardiac specific troponin T.

132. An assay according to claim 129, wherein said detectable signal is within a factor of 0.2 for equal amounts of said free cardiac specific troponin T and said complexed cardiac specific troponin T.

133. An assay according to claim 129, wherein said detectable signal is within a factor of 2 for equal amounts of said free cardiac specific troponin T and said complexed cardiac specific troponin T.

### Remarks

Applicants submit this preliminary amendment with an accompanying divisional patent application filed under 37 C.F.R. §1.53(b). The accompanying patent application is a divisional of co-pending U.S. Application Serial No. 08/633,248 filed April 18, 1996. The accompanying application is a true copy of U.S. Patent Application Serial No. 08/633,248.

The instant claims are fully supported by the specification and do not introduce new matter. The instant claims embody the subject matter of claims 101-148 of U.S. Application Serial No. 08/633,248, which were subject to a restriction requirement in an Office Action mailed on August 5, 1998.

Additionally, support for measurement of cardiac specific isoforms of troponin in patient samples, and particularly blood, serum, and plasma samples, can be found in the specification on page 10, lines 5-21.

Conclusion

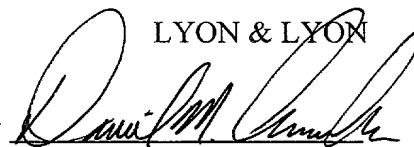
Applicants respectfully submit that the foregoing claims are in condition for allowance. An early notice to that effect is earnestly solicited. Should any matters remain outstanding, the Examiner is encouraged to telephone the undersigned at (619) 552-8400 so that they may be resolved without the need for additional action and response thereto.

Respectfully submitted,

LYON &amp; LYON

Dated 07/07/99

By



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Novel Methods for the Assay of Troponin I and T and Complexes  
of Troponin I and T and Selection of Antibodies for Use in  
Immunoassays

5      Cross-Reference to Related Applications

          This application is a continuation-in-part of U.S. Application serial No. 08/423,582, filed 18 April 1995, in the name of Kenneth F. Buechler and Paul H. McPherson, which is incorporated by reference herein.

10

Technical Field

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          This invention relates to the assay of troponin I and troponin T and complexes of these proteins, and more specifically to the changes in conformation of these proteins in blood, serum and plasma and to the selection of antibodies to the various forms of these proteins and their use in immunoassays. In another aspect of the invention, compositions are taught for the stabilization and recovery of troponin I and T and their complexes in immunoassays.

20

Background Art

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          Myocardial infarction is one of the leading causes of death in the United States. Approximately 5 million individuals experiencing chest pain are evaluated every year in hospitals throughout the United States, however, less than 30%, of these individuals are subsequently found to have had a myocardial infarction. The accurate and rapid diagnosis of myocardial infarction is important both for the patient suffering a myocardial infarction and for the health care system which can minimize the costs incurred by rapidly identifying individuals who do need treatment.

The diagnosis of myocardial infarction is usually performed in the emergency department of a hospital. An individual having the symptoms of myocardial infarction is treated in different ways depending on the obviousness of the condition. Generally, an electrocardiogram is given to assess the condition of the heart; however, approximately 50% of patients experiencing myocardial infarction have a non-diagnostic electrocardiogram. The physician is then faced with a problem of diagnosing and treating the patient suspected of having a myocardial infarction. Thus, diagnosis and treatment is difficult for patients with a suspected myocardial infarction who have non-diagnostic electrocardiograms.

The World Health Organization (WHO) has instituted guidelines for diagnosing myocardial infarction which state that an individual must exhibit two-of-the-three following criteria: 1) have chest pain or a history of cardiac disease; 2) a diagnostic electrocardiogram; and, 3) elevated creatine kinase (CK) or creatine kinase MB isoenzyme (CKMB). Thus, for the 50% of the individuals who are presented to hospitals for a suspected myocardial infarction and who have a non-diagnostic electrocardiogram, the physician must rely on symptoms of chest pain and an elevated CK or CKMB to diagnose a myocardial infarction.

The assay of CK or CKMB is generally performed in hospital laboratories using sophisticated instrumentation. The assays include enzyme assays and immunoassays which detect the activity or mass of CK or CKMB present in blood samples.

During a myocardial infarction, heart muscle cells die and release their contents to the blood stream. The CKMB is released among such cellular components. CKMB becomes

elevated above an otherwise nominal value and can be diagnostic for myocardial infarction. The specificity of CKMB for diagnosing myocardial infarction is not 100% because another source of CKMB in the body is skeletal muscle. Since the mass of skeletal muscle in the body far exceeds the mass of cardiac muscle, through the normal catabolic turnover of skeletal muscle cells, the blood concentration of CKMB in healthy individuals will vary. In general, the concentration of CKMB which may be indicative of myocardial infarction is above 5-7 ng/ml (Circulation 87, 1542-1550 (1993), Clin. Chem. 39, 1725-1728 (1993)). The CKMB concentration of individuals who have skeletal muscle injury or who have exercised has been reported to be elevated above 9 ng/ml (Clin. Chem. 38, 2396-2400 (1992)). Therefore, the problem of specificity when using CKMB as a marker for myocardial infarction has prompted the search for other more specific markers which are released only from damaged heart muscle.

Troponin I and troponin T have recently been shown to be more specific than CKMB for diagnosing myocardial infarction (Circulation 83, 902-912 (1991), Clin. Chem. 40, 1291-1295 (1994). Although troponin T has some disadvantages as a marker because it is elevated in patients experiencing renal disease (Clin. Chem. 41, 312-317 (1995)), the inventive methods herein disclose the successful use of troponin T as a diagnostic marker. The use of troponin I as a diagnostic marker for myocardial infarction also appears to meet many of the clinical requirements (Clin. Chem. 40, 1291-1295 (1994), Clin. Chem. 41, 312-317 (1995)).

The troponin complex in muscle is comprised of troponin I, C and T. These troponin components exist as various tissue specific isoforms. Troponin C exists as two isoforms, one from cardiac and slow-twitch muscle and one

from fast-twitch muscle. Troponin I and T are expressed as different isoforms in slow-twitch, fast-twitch and cardiac muscle (Biochem. J. 171, 251-259 (1978), J. Biol. Chem. 265, 21247-21253 (1990), Hum. Genet. 88, 101-104 (1991), Circul. Res. 62, 1226-1233 (1991)). The unique cardiac isoforms of troponin I and T allow them to be distinguished immunologically from the other troponins of skeletal muscle. Therefore, the release into the blood of troponin I and T from damaged heart muscle has been related to cases of unstable angina and myocardial infarction. The prior art, however, has not addressed other forms of troponin I and T in blood.

The troponin complex in muscle is tightly bound to the contractile apparatus. Approximately 6% of the troponin T in cardiac tissue exists as an unbound protein in the cytoplasm and it is believed that this pool of troponin T is released from damaged muscle (Am. J. Cardiol. 67, 1360-1367 (1991)).

The conformations of troponin I, T and C change upon binding when forming binary and ternary complexes (Biochemistry 33, 12800-12806 (1994), J. Biol. Chem. 254, 350-355 (1979), Ann. Rev. Biophys. Biophys. Chem. 16, 535-559 (1987)). An understanding of the conformational changes of troponin I and troponin T and the heterogeneity of the proteins in the blood is critical for the development of accurate diagnostic procedures for measuring troponin I and troponin T concentrations. In addition, troponin I is reported to be unstable in blood (Direction Insert for Troponin I Immunoassay, Sanofi/ERIA Diagnostics Pasteur, Marnes la Coquette, France), and the mechanisms responsible for the instability have not been understood. This invention

addresses these problems and provides for stable troponin I and T compositions which are useful in immunoassays.

5 The teachings of the instant invention provide methods for the selection of antibodies and their use in immunoassays for troponin I and troponin T and complexes of these proteins. These proteins, along with troponin C, exist in both cardiac and skeletal muscle mainly as a ternary complex. In the muscle, the troponin complex is bound to tropomyosin which is, in turn, bound to the actin comprising  
10 the thin filaments. The state of troponin I and troponin T, whether free or bound as binary or ternary complexes, which are released from the muscle, has not been previously investigated.

15 Disclosure of the Invention

Disclosed is an immunoassay system for determining the presence or amount of a troponin form or a group of troponin forms in a whole blood, plasma or serum sample suspected of containing troponin from damaged heart muscle.  
20 The system comprises: a) formation of an antibody conjugate comprising an antibody coupled to a signal generating element, said antibody capable of specifically binding to cardiac specific regions of a form of troponin or a group of troponin forms; b) formation of a reaction mixture comprising  
25 said whole blood, plasma or serum sample incubated with said antibody conjugate; c) application of said reaction mixture to a surface to which is bound at least one capture antibody capable of specifically binding to cardiac specific regions of a form of troponin or a group of troponin forms in said  
30 antibody conjugate, said capture antibody binding said antibody conjugate, whereby the immobilized conjugate produces a detectable signal upon formation of sandwich

complexes; and, d) relation of detectable signal to the presence or amount of said troponin form or said group of troponin forms in said sample.

Also disclosed are antibodies that are sensitive and antibodies that are insensitive to the form of troponin. An "antibody" refers to: a monoclonal antibody, a polyclonal antibody, a binding fragment of an antibody, a recombinant antibody, or a receptor protein that specifically binds to a target. As used herein, an insensitive antibody is an antibody that yields an assay response that is less than within about a factor of 2 (i.e., 50% of the base value); and preferably yields an assay response that is within about 20% for each form of troponin, measured relative to assay response for the use of that antibody in an assay for a troponin form or group of troponin forms (the base value). Thus, an insensitive antibody is one that will tend to bind more than one form of troponin.

As used herein, a sensitive antibody in an immunoassay is one that yields an assay response that is greater by at least about a factor of 2 larger (i.e., 200% of the base value) and preferably a factor of 5 larger (i.e., 200% of the base value), for one or a group of forms of troponin (the base value), as compared to the assay response for other forms measured. Thus, a sensitive antibody is one that will tend to bind only a single form of troponin.

As used herein the nine troponin forms are: 1) the cardiac ternary complex; 2) the cardiac troponin binary complex of I(oxidized)/T; 3) the cardiac troponin binary complex of I(reduced)/T; 4) the cardiac troponin binary complex of I(oxidized)/C; 5) the cardiac troponin binary complex of I(reduced)/C; 6) the cardiac troponin binary complex T/C; 7) unbound cardiac troponin I (oxidized); 8)



unbound cardiac troponin I (reduced); and, 9) unbound cardiac troponin T.

Disclosed is a stabilized composition of troponin; The stabilized composition can comprise a stabilized composition of troponin I, wherein the troponin I is oxidized, the troponin I can be unbound or the troponin I can be in a complex. The stabilized composition can comprise a stabilized composition of the ternary complex of troponin I, T and C.

Disclosed is a method for improving the recovery of troponin I or T from a surface used in immunoassays, said method comprising: contacting with said surface at least one strongly basic peptide, protein, or polymer with a pI value greater than about 8; the method can further comprise a step of washing unbound peptide, protein or polymer from said membrane. Melittin can be the strongly basic peptide used, protamine can be the strongly basic protein used.

#### Description of Figures

Figure 1a illustrates the kinetics of air oxidation of troponin I as measured by immunoassay.

Figure 1b illustrates the kinetics of oxidation by peroxide of troponin I as measured by immunoassay.

Figure 2 illustrates the kinetics of reduction by dithiothreitol of troponin I and reoxidation of reduced troponin I by peroxide as measured by immunoassay.

Figure 3 illustrates the effect of troponin C on the immunoassay of troponin I in the presence or absence of troponin T and binding inhibitors.

Figure 4 illustrates the kinetics of disruption of human cardiac troponin ternary complex in the presence or absence

of binding inhibitors as measured by an immunoassay for troponin I.

Figures 5a-5f illustrate the effect of binding inhibitors on troponin I immunoassays from patient samples with confirmed myocardial infarction.

#### Modes for Carrying Out the Invention

##### Definitions:

As used herein, an "antibody" or "receptor protein" refers to a monoclonal antibody, a polyclonal antibody, a binding fragment of an antibody, a recombinant antibody, or a receptor protein that specifically binds to a target. Specific binding of a substance signifies the quality of that substance that the substance will tend not bind to something to which it does not specifically bind; conversely, the substance will have greater affinity for something it specifically binds that for a something it does not specifically bind.

As used herein, an insensitive antibody in an immunoassay is an antibody that for each form of troponin of interest yields an assay response value that is the same within about a factor of 2, and preferably the same within about 20%, as the assay response values for the other forms of interest. Thus, an insensitive antibody is one that will exhibit a detection of more than one form of troponin in an immunoassay.

As used herein, a sensitive antibody in an immunoassay is one that for one form or group of forms of troponin yields an assay response value that is at least about a factor of 2 larger, and preferably, about a factor of 5 larger, than the assay response values for other forms. Thus, a sensitive antibody is one that will exhibit a

preferential detection of one form or group of forms of troponin in an immunoassay.

As used herein the nine troponin forms are: 1) the cardiac ternary complex; 2) the cardiac troponin binary complex of I(oxidized)/T; 3) the cardiac troponin binary complex of I(reduced)/T; 4) the cardiac troponin binary complex of I(oxidized)/C; 5) the cardiac troponin binary complex of I(reduced)/C; 6) the cardiac troponin binary complex T/C; 7) unbound cardiac troponin I (oxidized); 8) unbound cardiac troponin I (reduced); and, 9) unbound cardiac troponin T.

As used herein, a "zone" is a concept that correlates with the ability to identify distinct sensible signals. A zone, therefore, can correspond to a geographic region or correspond to the ability to separately identify distinct sensible signals. The sensible signals can be distinct by, but not limited to, variations between the following characteristics: wavelength of fluorescence or optical absorbance or reflectance; life time of, or transition energy between, electronic states; oxidation-reduction potentials; colorimetric characteristics; or, signal type (e.g., fluorescence vs. radioactivity vs. optical absorbance).

As used herein, unbound troponin is troponin that is not in a complex. A troponin complex can be binary or ternary.

As used herein, a "label", "signal generator" or "signal generating element" is an entity that can embody a number of different forms: Enzymes and their resultant effects on a substrate, colloidal metal particles, latex and silica particles with dye incorporated, and dye particles are examples of signal generators. An enzyme can react on a

substrate to produce a product that is sensible, for example, by wavelength of fluorescence (e.g., ultraviolet, visible, infrared), or sensible by affect on pH.

Modes:

5                   This invention is directed to the assay of troponin I and troponin T and complexes of these proteins in body fluids, particularly, in human blood, serum and plasma. The presence of cardiac troponin I and T in the blood, above a nominal concentration, is diagnostic for damaged heart muscle. The teachings of this invention show that troponin I and T exist in various conformations in the blood which may be the same or different than their native conformations in muscle tissue. These various conformations of the troponin molecules can react differently with antibodies.

10                   The ratios of the monomeric troponin I and T and the binary and ternary complexes may be related to the metabolic state of the heart. Based on the reactivities of antibodies to troponin I and T and to purified complexes of the troponins, the concentrations of troponin I and T and their complexes can now be elucidated in blood samples from patients suffering from myocardial infarction.

15                   The embodiments of this invention relate to the conformations of troponin I and T and their complexes in blood, serum and plasma, and to antibodies which recognize those conformations. Specifically, antibodies which recognize troponin I and T in the following forms are preferred: 1) The conformations of troponin I having intramolecularly oxidized and reduced cysteines; 2) The binary complexes of troponin I and T, of troponin I and C, of troponin T and C; and, 3) The ternary complex of troponin I, T and C. In addition, methods are described for the improved recovery of troponin I and T in immunoassays. This invention

answers the heretofore unmet need for the assays of troponin I and T in blood.

5           The focus on troponin I and T for use as markers for myocardial infarction has been based in part on their molecular size: because the proteins are relatively small, it is believed that they leak out of damaged cells faster than the larger proteins.

Antibodies to Troponin Complexes and to Troponin I and T

10           The term "antibodies or receptor proteins" as used herein refer to monoclonal and polyclonal antibodies, binding fragments of antibodies, and receptor proteins that specifically bend to a target. In a preferred embodiment, receptor proteins, for example, antibodies or binding fragments, are directed to the epitopes of troponin I which  
15           are insensitive to the oxidation state of the molecule. The terms sensitive and insensitive herein refer generally to the ability of an antibody to recognize particular forms of free troponin or troponin complexes. In particular, a sensitive antibody which is useful in an immunoassay distinguishes one  
20           form or forms of troponin from another form and an insensitive antibody which is useful in an immunoassay does not distinguish one form or forms of troponin from another. The sensitivity or insensitivity of an antibody is exhibited in an immunoassay. To determine whether an antibody is  
25           sensitive or insensitive, the antibody is tested with each troponin form independently to yield the assay response of the antibody for each troponin form. In general, a preferred antibody that is insensitive to the troponin form will yield an assay response that is the same, within about a factor of  
30           two and preferably within 20%, for each form of troponin. A preferred antibody that is sensitive to the troponin form will yield an assay response that is at least a factor of

two, and preferably a factor of five, larger for one form or group of forms as compared with the other form(s). In addition, the terms "troponin I and T" can refer to the free, uncomplexed troponins or to the troponins in the binary or ternary complexes. Human cardiac troponin I contains two cysteines, at positions 80 and 97 (FEBS Letters, 270, 57-61 (1990)). In the current art, during the purification of troponin I from tissues, the oxidation state of troponin I is directed toward the reduced form using various reductants, including mercaptoethanol, dithiothreitol and the like (Can. J. Biochem. 54, 546-553 (1976), Methods Enzymol. 85, 241-263 (1982)). After purification, the current art also teaches to maintain troponin I in the reduced form to prevent intermolecular disulfide formation (J. Biol. Chem. 258, 2951-2954 (1983)).

In the development of immunoassays for a target protein, the purified target protein acts as a standard with which to judge the sensitivity and specificity of the immunoassay using the antibodies that have been selected. As disclosed herein, the cysteines in troponin I can rapidly oxidize, intramolecularly, to alter the conformation of the protein. The degree of oxidation of troponin I has not previously been addressed with respect to its effect on the immunoassay process. The teachings described herein show that an apparent instability in the troponin I molecule is related to the dynamics of the intramolecular oxidation or reduction of the troponin I molecule. In addition, the selection of antibodies is taught for the accurate quantitation of troponin I in blood.

Purified, reduced troponin I undergoes an intramolecular oxidation of the cysteines, the rate of which is not dependent on the troponin I concentration. Special

care should be exercised when preparing the oxidized troponin I form, especially in the presence of various thiol reducing agents, because of the possibility of forming mixed disulfides of the protein and the reducing thiol reagent.

5 The mixed disulfide form of the protein may not behave as either the oxidized or reduced form of the molecule, especially if the antibodies used in the immunoassay bind to the region of the protein surrounding cysteines 80 and 97.

10 Using purified preparations of oxidized and reduced troponin I, differential effects in the immunoassays using various antibodies raised to troponin I were observed. With some antibody pairs, the reduced troponin I was hardly detectable in immunoassays, whereas with other pairs, the oxidation state had no effect on the immunoassay process.

15 These results showed that selection of antibodies to the troponin I molecule, without prior knowledge of the oxidation state of the troponin I, can result in antibodies and an immunoassay process which gives erroneous results. This conclusion is exemplified by immunoassays of troponin I from

20 patients suffering myocardial infarction. The degree of oxidation of troponin I in patient samples is variable and suggests a possible basis for apparent instabilities of troponin I assays of the current art. The free troponin I does change its oxidation state from the reduced to the

25 oxidized form over time (see Example 4).

In the case when an immunoassay comprising an insensitive antibody is to be utilized to bind to the free and complexed troponin, a preferred antibody is one that is insensitive with respect to the oxidized, reduced and complex

30 forms because the circulating troponin in the blood can change its oxidation state and the degree of binding to other troponin components over time. For example, if reduced, free

troponin I is released from the heart, it can transform to the oxidized form during circulation in the blood and until the time troponin is measured. In addition, the free troponin I and T in the blood can bind to each other and to troponin C to form binary and ternary complexes. The outcome of these transformations to the oxidized form of troponin I or to complexes of troponin is that when an antibody is chosen for an immunoassay that is sensitive with respect to the oxidized and reduced forms and complexed forms, the assay will show an apparent increase or decrease in the troponin concentration over time depending on which form (free, oxidized or reduced or complexed) the antibody better recognizes, rather than an actual change in troponin concentration. In another example, the release of troponin complexes from damaged heart muscle can result in the formation of free (uncomplexed) and binary forms of troponin I and T during circulation in the blood or until the troponin is assayed. The variability of assay results of troponin concentrations using immunoassays comprising a sensitive antibody or antibodies can mislead a physician into believing that a patient's condition is improving or deteriorating.

In the case when an immunoassay comprising more than one sensitive antibody is used to measure the free and complexed troponin, each sensitive antibody is intended to be reacted with one form or group of forms of troponin such that the antibody exhibits a maximum assay response for the intended form(s) and a minimum assay response for all other forms. Preferred antibodies are ones for which the assay responses for the intended forms are about the same, preferably within 20%, for all the sensitive antibodies and said minimum assay responses are at least a factor of 2 and preferably a factor of 5 less than the maximum assay



responses. For example, if two different antibodies are used for signal antibodies in an immunoassay, and one antibody is insensitive with respect to the oxidized and reduced free troponin I (or the free troponin T) and exhibits a maximum assay response for said free forms of troponin and a minimum assay response for the complexed troponin, then the other antibody should exhibit a maximum assay response for the complexed troponin and a minimum assay response for the free troponin I and T forms. In this way, an accurate measure of total troponin can be determined. One skilled in the art will also recognize that antibodies with different affinities that is, exhibit different assay responses, for the troponin forms can also be utilized in immunoassays when each troponin form is measured alone or in discrete zones and that the relative bias of the immunoassays can be accounted for in the calibration of the assay. Also, sensitive and insensitive antibodies can be attached to solid phases to measure each troponin form in discrete zones.

In another preferred embodiment, antibodies or binding fragments that are directed to the epitopes of the troponin I or T are insensitive with respect to free troponin I or T and troponin complexes. The teachings herein show that antibodies that are sensitive with respect to free troponin I or T and troponin complexes provide methods for the estimation of the extent of binding of troponin I or T in complexes. Using purified preparations of troponin I, T and C, and the purified troponin complexes, the effects of troponin I or T binding in complexes on the recognition of troponin by antibody pairs is taught and is related to the dynamic state of troponin I or T in blood.

The degree of binding of troponin I and T to the components of the troponin complex or to other proteins of

the contractile apparatus, including tropomyosin and actin, in blood can also be problematic for immunoassays depending on the degree and affinity of binding. In their native forms, the troponin complex exists in cardiac muscle and in  
5 slow- and fast-twitch skeletal muscle as a ternary complex of troponin I, C and T. Troponin I and T from skeletal muscle have different amino acid sequences than troponin I and T from cardiac muscle, respectively; however, troponin C from slow-twitch muscle has the same amino acid sequence as the  
10 cardiac muscle protein (Nature 271, 31-35 (1978), Arch. Biochem. Biophys. 186, 411-415 (1978), FEBS Lett. 292, 5-8 (1991)). The fast-twitch skeletal muscle troponin C, although not identical to the cardiac troponin C, can bind to cardiac troponin I (Biochemistry 33, 8464-8471 (1994), Proc.  
15 Natl. Acad. Sci. USA 90, 9036-9040 (1993)).

The release of troponin components, that is, troponin I, C and T, or components from the contractile apparatus, for example, tropomyosin and actin, from skeletal muscle, due to the normal turnover of skeletal muscle cells,  
20 may result in a significant amount of troponin and contractile apparatus components in the blood. Since skeletal muscle mass is much greater than cardiac muscle mass, the troponin components present in the blood of a normal individual may be derived largely from skeletal  
25 muscle. The circulating troponin components which are mainly derived from skeletal muscle would bind to cardiac troponin I and T which are released into the blood during a myocardial infarction or events which lead up to creating damaged heart muscle. As muscle damage progresses in an individual the  
30 troponin components derived from heart tissue will presumably rise in the blood. Thus, the concentration of troponin components (bound and free) in the blood from individuals

experiencing a myocardial infarction may be differentially derived from both cardiac and skeletal muscle.

The form of troponin released from the heart, whether free or as binary or ternary complexes, into the blood may indicate a particular condition of the heart. The assays taught herein provide for the analysis of release patterns which may allow the physician to diagnose a specific heart failure, for example, unstable angina as compared to myocardial infarction or to determine the time that an infarction occurred.

The clinical impact of an immunoassay measuring only the free troponin I or T from a patient experiencing a myocardial infarction can be very significant. Since the binding of troponin I and T to troponin components in the blood will be variable, depending on the troponin component concentrations, and on the time that has elapsed since the release of the troponin components from muscle an analysis of the bound and free form of the troponin I and T in the blood must be considered. For example, the binding affinity of troponin I to troponin C, in the presence of calcium (which also is present in blood) is  $1.27 \times 10^8 \text{ M}^{-1}$  (Biochemistry 33, 12729-12734 (1994)). This implies that if the troponin C concentration is 100 ng/ml and the total (bound and free) troponin I concentration is 8 ng/ml, then the free concentration of troponin I is calculated to be 4.6 ng/ml. If the concentration of troponin I which is indicative of a myocardial infarction is 5 ng/ml or greater, then an assay which measures only the free form of troponin I, in this case, 4.6 ng/ml, will indicate to the physician that a myocardial infarction has not taken place. Generally, in hospital emergency departments which admit patients believed to have had a myocardial infarction, a blood sample from the

individual will be obtained again in an hour or two if the first result is negative. In this example, the patient, having a total troponin I concentration of 8 ng/ml in the first sample, (which is defined as positive for a myocardial infarction), but only a measured concentration of 4.6 ng/ml, (which would be defined as a negative result), would not be treated and would continue to accrue damaged heart muscle during the time before a second sample was analyzed.

Interpretation of results of troponin T assays would also suffer from troponin T binding to components of the contractile apparatus in blood. Thus, immunoassays of the current art that measure the free troponin I and T may not correctly diagnose a myocardial infarction when the troponin I or T concentration, respectively, is near the decision point. In some cases, the rise or fall of the troponin I or T concentration in a patients blood over time as determined by analyzing blood samples drawn at several different times might be used to diagnose the dynamic condition of the heart, for example, to determine whether the damaged heart is improving with therapy or continuing to deteriorate. In these cases, the time-course of the free troponin I or T concentration could be different than that of the total troponin (bound and free) concentration. One skilled in the art will realize that increasing concentrations of all the troponin components in the blood will result in an increasing fraction of bound troponin I and T relative to free troponin. The concentration of total troponin (bound and free) may rise faster than the concentration of free troponin I and T. An assay that measures the total troponin concentration (bound and free troponin I and T) would be more accurate in assessing progression of heart damage as compared with an assay that measures only free troponin I or T.

In a particularly preferred embodiment, antibodies or binding fragments are directed to the cardiac troponin complex. Specifically, antibodies are directed to cardiac specific epitopes of troponin I and T of the troponin complex or of the troponin I/T, I/C and T/C interfaces in the complex. The teachings herein show that antibodies that are raised to troponin I and T can bind poorly to troponin I or T of the ternary complex. Furthermore, the teachings herein show that the troponin complex exists in the blood of patients who have experienced myocardial infarction. Methods are also described which teach one skilled in the art to assess the amount of troponin complex in the blood relative to the free troponin I and T or binary complexes of troponin I and T, using antibodies which bind to the free troponin molecules.

The equilibrium among the binary and ternary complexes of troponin and free troponin I and T will be altered during the immunoassay process because of the binding of antibodies to the troponin components and complexes. The change in the mole fractions of the various species during the immunoassay may be significant or insignificant and will be a function of the antibody concentrations, the affinity of the antibodies for the troponin components and complexes, the association constants for the troponin components and complexes and the time that the antibodies are allowed to bind to the troponin. These variables can change the perceived concentration of troponin I and T and lead to erroneous conclusions about the troponin concentration. For example, if two immunoassays utilize different antibody pairs for performing a sandwich immunoassay and their antibody concentrations and affinities for troponin I or T are different, and if a proportion of the troponin I or T occurs

in the sample as binary and ternary complexes, one may expect that each immunoassay will give a different result. In addition, if blood samples contain varying concentrations of troponin C, then the proportion of troponin I and T that is bound to troponin C as a binary complex will differentially perturb each immunoassay.

The teachings of the instant invention demonstrate that the troponin ternary complex is more stable to dissociation than the binary complexes of troponin.

In another preferred embodiment, antibodies or binding fragments are directed to epitopes which are not changed by proteolytic degradation of the N-terminal region of troponin I. The conformation of troponin I is also reported to be affected by phosphorylation/dephosphorylation (Biophys. J. 63, 986-995(1992), Biochem. 33, 12729-12734 (1994)). In another preferred embodiment, antibodies or binding fragments are directed to epitopes of either troponin I or troponin I complexes, which are or are not changed by the phosphorylation state of troponin I. Troponin I can be phosphorylated using methods described in J. Biol. Chem. 252, 851-857 (1977). The phosphorylated and dephosphorylated preparations of troponin I can be utilized as immunogens for generating antibodies as well as antigens for the selection of antibodies to the phosphorylated and dephosphorylated troponin I. The troponin complex can be dissociated into the component proteins using various treatments, including high concentrations of urea, low pH and metal chelating agents which bind divalent metal cations, particularly calcium and magnesium (Methods Enzymol. 85, 241-263 (1982)). These treatments are, in general, very harsh and require several hours. Thus, these conditions for dissociating the troponin complex are not practical for immunoassays which

must be performed in a matter of minutes on samples from individuals who may be suffering a myocardial infarction.

The generation and selection of antibodies that are preferentially either sensitive or insensitive to the binding of troponin I or T in binary complexes are accomplished by first preparing binary troponin I/T, T/C and I/C complexes from purified components (J. Biol. Chem. 254, 350-355 (1979), J. Biol. Chem. 258, 2534-2542 (1983), J. Biol. Chem. 258, 2951-2954 (1983), Can. J. Biochem. Cell Biol. 63, 212-218 (1985), Biochemistry 33, 12729-12734 (1994), Ann. Rev. Biophys. Biophys. Chem. 16, 535-559 (1987)). The complexes may be stabilized, if necessary, by chemically cross-linking the proteins in the complex using methods familiar to those skilled in the art. The generation and selection of antibodies that are sensitive or insensitive to the binding of troponin I or T in the ternary complex can be accomplished several ways. For example, one way is to purify the ternary complex (Methods Enzymol. 85, 241-263 (1983)) or to reconstitute the complex using the purified troponin components. One skilled in the art will recognize that various other contractile apparatus proteins which may be associated with the binary or ternary complexes of troponins can also be constructed from the purified components and that the resultant complex can be utilized to generate and select antibodies as taught by the instant invention. The complex can be stabilized with respect to dissociation by chemically crosslinking the components. The purified complexes are then injected, for example, into mice or rabbits, to generate polyclonal or monoclonal antibodies. Another way is to purify free (unbound) troponin I or T and then inject the purified free troponin I or T, for example, into mice or rabbits, to generate polyclonal or monoclonal antibodies.

One skilled in the art will recognize that many procedures are available for the production of antibodies, for example, as described in Antibodies, A Laboratory Manual, Ed Harlow and David Lane, Cold Spring Harbor Laboratory (1988), Cold Spring Harbor, NY. One skilled in the art will also appreciate that binding fragments or Fab fragments which mimic antibodies can also be prepared from genetic information by various procedures (Antibody Engineering: A Practical Approach (Borrebaeck, C., ed.), 1995, Oxford University Press, Oxford; J. Immunol. 149, 3914-3920 (1992)). In particular, the preparation, screening and selection of recombinant binding fragments is described in Examples 22 and 23.

The antibodies which are generated are selected by first screening for affinity and specificity with the purified binary or ternary complexes and comparing the results to the affinity and specificity of the antibodies with the purified troponin I and T molecules for the desired properties which are defined by the immunoassay process.

The screening procedure can involve immobilization of the purified troponin I or T or binary or ternary complexes or peptides to cardiac specific sequences of the troponins in separate wells of microtiter plates. The solution containing a potential antibody or groups of antibodies is then placed into the respective microtiter wells and incubated for about 30 min to 2 h. If an antibody to the protein of interest is present in the solution, it will bind to the immobilized troponin. In screening antibodies for binding to interfaces of binary or ternary complexes of troponin, an antibody is first selected which binds to the binary or ternary complex immobilized in the microtiter well. That antibody is then further screened for



its ability to bind to free troponin components; that is, the potential interface antibody should not bind to free troponin I, C or T which is immobilized in microtiter wells. In addition, the interface antibody should never be capable of forming a sandwich assay with binary or ternary complexes and an antibody which is known to bind to a specific troponin component in the complex in the presence of binding inhibitors which are known to disrupt the troponin complex. If this latter condition is met, then the potential interface antibody should also not be capable of forming a sandwich assay with the troponin complex and an antibody to a different troponin component than was used in the previous screen in the presence of binding inhibitors. If this condition is also met, then an interface antibody has been selected for a binary complex. An extra immunoassay must be performed for selecting an interface antibody to a ternary complex; that is, if the previous two conditions are met, then the potential interface antibody should also not be capable of forming a sandwich assay with the troponin complex and an antibody to a different troponin component than was used in the two previous screens. The microtiter wells are then washed and a labeled secondary antibody (for example, an anti-mouse antibody conjugated to alkaline phosphatase if the raised antibodies are mouse antibodies) is added to the wells and incubated for about 30 min and then washed. Substrate is added to the wells and a color reaction will appear where antibody to the troponin is present. The antibodies which are of interest are then further analyzed for affinity and specificity to the cardiac specific molecules and for complementarity in forming sandwich complexes with the antigens. Those skilled in the art will recognize that many approaches can be taken in producing antibodies or binding

fragments and screening and selecting for affinity and specificity for the various troponin antigens, but these approaches do not change the scope of the invention.

5     Assays for Troponin Complexes and Uncomplexed Troponin I and T

          A particularly preferred embodiment of this invention is directed to the assay of troponin I and troponin T, particularly immunoassays, wherein the antibodies selected  
10     for the assay bind to cardiac specific sequences of the ternary complex, of the binary complexes and of the uncomplexed (free) troponin I or T in order to measure the complexed (bound) and free fractions of troponin I and T, respectively. The cardiac specific sequences of troponin I  
15     and T are described in FEBS Lett. 270, 57-61 (1990) and Genomics 21, 311-316 (1994). A synthetic peptide comprised of 14 amino acids which mimics a cardiac specific sequence of troponin I and methods used to prepare antibodies to the peptide are described in an International Patent Application  
20     number PCT/US94/05468.

          The immunoassay can be formulated with a cocktail of antibodies to bind all the troponin complexes and the free troponin I and T. Alternatively, the immunoassay can be formulated with specific antibodies that recognize epitopes  
25     of the troponin I and T in the complexes and also the unbound troponin I and T. In addition, the immunoassay can be formulated with antibodies that bind epitopes at interfaces of the component proteins in the complexes and antibodies that bind the unbound troponin I and T.

30     A preferred immunoassay for troponin I or T involves conjugation of an antibody or a cocktail of antibodies to a label or a signal generator to form an

antibody conjugate(s), which are capable of binding to cardiac specific regions of the troponin complexes of troponin I or T and to unbound troponin I or T. One skilled in the art will recognize that a signal generator has many forms. Enzymes, colloidal metal particles, latex and silica particles with dye incorporated, and dye particles are examples of signal generators. Antibodies can be conjugated to the signal generators in a variety of ways using heterobifunctional reagents as taught in the Pierce Catalog and Handbook, Pierce Chemical Co., Rockford, IL and in Uniform Latex Particles by Leigh B. Bangs, Seragen Diagnostics Inc., Indianapolis, IN hereby incorporated by reference. Another antibody or cocktail of antibodies is immobilized on a solid phase, for example, a membrane as taught in BioTechniques 4, 272-283 (1986), and the membrane is placed in a device, for example, as described in U.S. Patents, 4,727,019 and 5,458,852. The immobilized antibody is complementary to the antibody conjugate. The immobilized antibody and conjugate antibodies form sandwich complexes with the troponin I or T complexes and also form sandwich complexes with troponin I or T, respectively. A plasma or serum sample suspected of containing troponin complexes or components from damaged heart muscle is mixed with the antibody conjugate to form a reaction mixture that is allowed to incubate. The reaction mixture is then applied to the aforementioned device. The sample flows through the membrane and the troponin complexes and components, bound to the antibody conjugates, bind to the immobilized antibodies, and excess, unbound, antibody conjugate is washed away with a wash buffer. The signal is developed and read, either visually or instrumentally.

A particularly preferred immunoassay for troponin I involves conjugation of at least two antibodies to a label or a signal generator to form an antibody conjugate. One of the conjugate antibodies is capable of binding to the troponin T component of the troponin ternary complexes and the other antibody is capable of binding to the free and binary troponin I molecules. Another antibody or cocktail of antibodies is immobilized on a solid phase, for example, a membrane, and the membrane is placed in a device, as described previously. The immobilized antibody is complementary with the antibody conjugate antibodies to form sandwich complexes with either troponin I bound to troponin complexes or to the uncomplexed troponin I. A plasma or serum sample suspected of containing troponin complexes or components from damaged heart muscle is mixed with the antibody conjugate to form a reaction mixture which is allowed to incubate. The reaction mixture is then applied to the aforementioned device. The sample flows through the membrane and the troponin complexes and components, bound to the antibody conjugates, bind to the immobilized antibodies and excess, unbound antibody conjugate is washed away with a wash buffer. The signal is developed and read, either visually or instrumentally. In this assay procedure, the antibody conjugate binds to the troponin I in ternary complexes through the troponin T specific antibody and all free and binary troponin I molecules through the troponin I specific antibody. The capture antibody or antibodies on the solid phase bind antibody conjugates that are bound to free troponin I, and to troponin ternary complexes that contain troponin I.

A particularly preferred immunoassay for troponin I (oxidized and reduced) involves conjugation of at least two

antibodies to a label or a signal generator to form an antibody conjugate. One of the conjugate antibodies is capable of binding to the oxidized troponin I and the other antibody is capable of binding to the reduced troponin I molecules. Another antibody or cocktail of antibodies is immobilized on a solid phase in up to 2 discrete zones, for example, a membrane, and the membrane is placed in a device, as described previously. The immobilized antibody is complementary with the antibody conjugate antibodies to form sandwich complexes with either oxidized troponin I or reduced troponin I. A plasma or serum sample suspected of containing troponin components from damaged heart muscle is mixed with the antibody conjugate to form a reaction mixture which is allowed to incubate. The reaction mixture is then applied to the aforementioned device. The sample flows through the membrane and the oxidized and reduced troponin I, bound to the antibody conjugates, bind to the immobilized antibodies and excess, unbound antibody conjugate is washed away with a wash buffer. The signal is developed and read, either visually or instrumentally. In this assay procedure, the antibody conjugates bind to the oxidized troponin I through the oxidized troponin I specific antibody and to the reduced troponin I through the reduced troponin I specific antibody. The capture antibody or antibodies on the solid phase only bind antibody conjugates that are bound to oxidized and reduced troponin I. This immunoassay may have application in the estimation of time after an infarction has occurred.

Another particularly preferred immunoassay for troponin I involves conjugation of an antibody or a cocktail of antibodies to a label or a signal generator to form an antibody conjugate. The antibody conjugate binds to either

troponin I bound to troponin complexes or to the uncomplexed troponin I. Immobilized on a solid phase, for example, a membrane, in 3 discrete zones, are antibodies or cocktails of antibodies which bind the ternary complex, the binary complexes of troponin I and the free troponin I, and the membrane is placed in a device, as described previously. For example, a troponin T antibody which binds to the troponin T of the ternary complex is immobilized in one discrete zone, a troponin I antibody which binds to the troponin I binary complexes (troponin I/C and I/T) is immobilized in another discrete zone and a troponin I antibody which binds to only the uncomplexed troponin I is immobilized in yet another discrete zone. The immobilized antibodies are complementary with the antibody conjugate antibodies to form sandwich complexes with complexed or uncomplexed troponin I, as defined by each discrete zone. Alternatively, immobilized on a solid phase, for example, a membrane, in 2 discrete zones, are antibodies or cocktails of antibodies which bind the troponin I complexes (binary and ternary) and the free troponin I. For example, a troponin T antibody which binds to the troponin T of the ternary complex and a troponin I antibody which binds to the troponin I of the binary complexes are immobilized in one discrete zone and a troponin I antibody which binds to only the uncomplexed troponin I is immobilized in the second discrete zone. The immobilized antibodies are complementary with the antibody conjugate antibodies to form sandwich complexes with complexed or uncomplexed troponin I, as defined by each discrete zone. A further embodiment of this invention utilizes antibodies on the solid phase for detection of troponin I complexes which bind to the interfaces of the binding domains of troponin I/T and I/C. A plasma or serum sample suspected of containing

troponin complexes or components from damaged heart muscle is mixed with the antibody conjugate to form a reaction mixture which is allowed to incubate. The reaction mixture is then applied to the aforementioned device. The sample flows  
5 through the membrane and the troponin complexes and components, bound to the antibody conjugate(s); bind to the respective immobilized antibodies in the discrete zones and excess, unbound antibody conjugate is washed away with a wash buffer. The signal is developed and read, either visually or  
10 instrumentally. In this assay procedure, the antibody conjugate binds to the troponin I and the troponin I binary and ternary complexes through the troponin I specific antibody or antibodies. The capture antibody or antibodies in discrete zones on the solid phase bind the antibody  
15 conjugates that are bound to the uncomplexed troponin I or troponin complexes containing troponin I as defined by each discrete zone. This immunoassay allows quantification of the fractions of troponin I, namely, the complexed and the uncomplexed fractions. The inventive teachings described  
20 herein show that uncomplexed and complexed troponin exists in plasma and serum samples from patients with confirmed myocardial infarction. The determination of the complexed and uncomplexed troponin I fractions may yield important clinical data relating to the type and extent of muscle  
25 damage, for example, from unstable angina or myocardial infarction or to the success of thrombolytic therapy.

Another particularly preferred immunoassay measures the cardiac troponin ternary complex, the cardiac troponin binary complexes (troponin I/T, T/C and I/C) and the free  
30 cardiac troponin I and T. This method involves conjugation of antibodies or a cocktail of antibodies to a label or a signal generator to form an antibody conjugate. The antibody

conjugates bind to either troponin T and I bound to troponin complexes or to the uncomplexed troponin T and I.

Immobilized on a solid phase, for example, a membrane, in a discrete zone, are antibodies or cocktails of antibodies

5 which bind the ternary complex, the binary complexes of troponin I and T and the free troponin I and T, and the membrane is placed in a device, as described previously. For

example, a troponin I antibody which binds to the troponin I of the ternary complex, up to 3 different antibodies, each

10 recognizing the interfaces of the binding domains of troponin I/T, T/C and I/C, a troponin I antibody which binds to the free troponin I and a troponin T antibody which binds to the

free troponin T are immobilized in a discrete zone. The immobilized antibodies are complementary with the antibody

15 conjugate antibodies to form sandwich complexes with complexed and uncomplexed troponin T and I. A plasma or serum sample suspected of containing troponin complexes or

components from damaged heart muscle is mixed with the antibody conjugate to form a reaction mixture and it is

20 allowed to incubate. The reaction mixture is then applied to the aforementioned device. The sample flows through the membrane and the troponin complexes and components, bound to the antibody conjugate(s), bind to the respective immobilized

antibodies in the discrete zone and excess, unbound antibody conjugate is washed away with a wash buffer. The signal is

25 developed and read, either visually or instrumentally. In this assay procedure, the antibody conjugates bind to the free troponin I and T, to the troponin I and T of the binary complexes and the troponin I or T of the ternary complex.

30 The capture antibodies in the discrete zone on the solid phase bind the antibody conjugates which are specific to the free troponin I and T and to the troponin complexes. This



immunoassay allows quantification of the ternary troponin complex, the troponin T/C, I/T, I/C and the free troponin I and T. One skilled in the art will recognize that the antibodies specific to the various troponin forms can be conjugated to one or more signal generators to form antibody conjugates and the antibodies previously described for the conjugates can be attached to the solid phase in a discrete zone. The determination of the total troponin concentration may yield a more sensitive immunoassay for damage to the heart muscle that will, in turn, allow a more rapid diagnosis of unstable angina or myocardial infarction and therefore faster administration of thrombolytic therapy.

A particularly preferred immunoassay for troponin T involves conjugation of at least two antibodies to a label or a signal generator to form an antibody conjugate. One of the conjugate antibodies is capable of binding to the troponin I component of the troponin complexes and the other antibody is capable of binding to the free and binary troponin T molecules. Another antibody or cocktail of antibodies is immobilized on a solid phase, for example, a membrane, and the membrane is placed in a device, as described previously. The immobilized antibody is complementary with the antibody conjugate antibodies to form sandwich complexes with either troponin T bound to troponin complexes or to the uncomplexed troponin T. A plasma or serum sample suspected of containing troponin complexes or components from damaged heart muscle is mixed with the antibody conjugate to form a reaction mixture and it is allowed to incubate. The reaction mixture is then applied to the aforementioned device. The sample flows through the membrane and the troponin complexes and components, bound to the antibody conjugates, bind to the immobilized antibodies and excess, unbound antibody conjugate

is washed away with a wash buffer. The signal is developed and read, either visually or instrumentally. In this assay procedure, the antibody conjugate binds to the troponin complexes through the troponin I specific antibody and all  
5 free and binary troponin T molecules through the troponin T specific antibody. The capture antibody or antibodies on the solid phase bind antibody conjugates that are bound to free troponin T, and to troponin complexes containing troponin T.

Another particularly preferred immunoassay for  
10 troponin T involves conjugation of an antibody or a cocktail of antibodies to a label or a signal generator to form an antibody conjugate. The antibody conjugate binds to either troponin T bound to troponin complexes or to the uncomplexed troponin T. Immobilized on a solid phase, for example, a  
15 membrane, in 3 discrete zones, are antibodies or cocktails of antibodies which bind the ternary complex, the binary complexes of troponin T and the free troponin T, and the membrane is placed in a device, as described previously. For example, a troponin I antibody which binds to the troponin I  
20 of the ternary complex is immobilized in one discrete zone, a troponin T antibody which binds to the troponin T binary complexes (troponin I/T and C/T) is immobilized in another discrete zone and a troponin T antibody which binds to only the uncomplexed troponin T is immobilized in yet another  
25 discrete zone. The immobilized antibodies are complementary with the antibody conjugate antibodies to form sandwich complexes with complexed or uncomplexed troponin T, as defined by each discrete zone. Alternatively, immobilized on a solid phase, for example, a membrane, in 2 discrete zones,  
30 are antibodies or cocktails of antibodies which bind the troponin T complexes (binary and ternary) and the free troponin T. For example, a troponin I antibody which binds

to the troponin I of the ternary complex and a troponin T antibody which binds to the troponin T of the binary complexes are immobilized in one discrete zone and a troponin T antibody which binds to only the uncomplexed troponin T is immobilized in the second discrete zone. The immobilized antibodies are complementary with the antibody conjugate antibodies to form sandwich complexes with complexed or uncomplexed troponin T, as defined by each discrete zone. A further embodiment of this invention utilizes antibodies on the solid phase for detection of troponin T complexes which bind to the interfaces of the binding domains of troponin C/T and I/T. A plasma or serum sample suspected of containing troponin complexes or components from damaged heart muscle is mixed with the antibody conjugate to form a reaction mixture which is allowed to incubate. The reaction mixture is then applied to the aforementioned device. The sample flows through the membrane and the troponin complexes and components, bound to the antibody conjugate(s), bind to the respective immobilized antibodies in the discrete zones and excess, unbound antibody conjugate is washed away with a wash buffer. The signal is developed and read, either visually or instrumentally. In this assay procedure, the antibody conjugate binds to the troponin T and the troponin T binary and ternary complexes through the troponin T specific antibody or antibodies. The capture antibody or antibodies in discrete zones on the solid phase bind the antibody conjugates that are bound to the uncomplexed troponin T or troponin complexes containing troponin T as defined by each discrete zone. This immunoassay allows quantification of the fractions of troponin T, namely, the complexed and the uncomplexed fractions. The inventive teachings described herein show that uncomplexed and complexed troponin exists in

plasma and serum samples from patients with confirmed myocardial infarction. The determination of the complexed and uncomplexed troponin T fractions may yield important clinical data relating to the type and extent of muscle damage, for example, from unstable angina or myocardial infarction or to the success of thrombolytic therapy.

Another particularly preferred immunoassay independently measures the cardiac troponin ternary complex, the cardiac troponin binary complexes (troponin I/T, T/C and I/C) and the free cardiac troponin I and T. This method involves conjugation of antibodies or a cocktail of antibodies to a label or a signal generator to form an antibody conjugate. The antibody conjugates bind to either troponin T and I bound to troponin complexes or to the uncomplexed troponin T and I. Immobilized on a solid phase, for example, a membrane, in 6 discrete zones, are antibodies or cocktails of antibodies which bind the ternary complex, the binary complexes of troponin I and T and the free troponin I and T, and the membrane is placed in a device, as described previously. For example, a troponin I antibody which binds to the troponin I of the ternary complex is immobilized in one discrete zone, 3 different antibodies, each recognizing the interfaces of the binding domains of troponin I/T, T/C and I/C, are immobilized in 3 discrete zones, a troponin I antibody which binds to the free troponin I is immobilized in another zone and a troponin T antibody which binds to the free troponin T is immobilized in another zone. The immobilized antibodies are complementary with the antibody conjugate antibodies to form sandwich complexes with complexed and uncomplexed troponin T and I, as defined by each discrete zone. A plasma or serum sample suspected of containing troponin complexes or components from damaged

heart muscle is mixed with the antibody conjugate to form a reaction mixture and it is allowed to incubate. The reaction mixture is then applied to the aforementioned device. The sample flows through the membrane and the troponin complexes and components, bound to the antibody conjugate(s), bind to the respective immobilized antibodies in the discrete zones and excess, unbound antibody conjugate is washed away with a wash buffer. The signal is developed and read, either visually or instrumentally. In this assay procedure, the antibody conjugates bind to the free troponin I and T, to the troponin I and T of the binary complexes and the troponin I or T of the ternary complex. The capture antibodies in discrete zones on the solid phase bind the antibody conjugates which are specific to the free troponin I and T and to the troponin complexes. This immunoassay allows quantification of the ternary troponin complex, the troponin T/C, I/T, I/C and the free troponin I and T. The inventive teachings described herein show that uncomplexed and complexed troponin exists in plasma and serum samples from patients with confirmed myocardial infarction. The determinations of the individual troponin ternary complex, the troponin I and T binary complexes and uncomplexed troponin I and T fractions may yield important clinical data relating to the type and extent of muscle damage, for example, from unstable angina or myocardial infarction or to the success of thrombolytic therapy.

Another particularly preferred immunoassay independently measures the cardiac troponin ternary complex, the cardiac troponin binary complexes (troponin I/T, T/C, I/C, oxidized I/T and oxidized I/C) and the free cardiac troponin I (oxidized and reduced) and T. This method involves conjugation of antibodies or a cocktail of

antibodies to a label or a signal generator to form an antibody conjugate. The antibody conjugates bind to either troponin T and I bound to troponin complexes or to the uncomplexed troponin T and I. Immobilized on a solid phase, for example, a membrane, in up to 9 discrete zones, are antibodies or cocktails of antibodies which bind the ternary complex, the binary complexes of troponin I and T and the free troponin I and T, and the membrane is placed in a device, as described previously. For example, a troponin I antibody which binds to the troponin I of the ternary complex is immobilized in one discrete zone, 5 different antibodies, each recognizing the interfaces of the binding domains of troponin I/T, T/C, I/C, oxidized I/T and oxidized I/C are immobilized in up to 5 discrete zones, a troponin I antibody which binds to the free troponin I (oxidized and reduced) is immobilized in up to 2 zones and a troponin T antibody which binds to the free troponin T is immobilized in another zone. The immobilized antibodies are complementary with the antibody conjugate antibodies to form sandwich complexes with complexed and uncomplexed troponin T and I, as defined by each discrete zone. A plasma or serum sample suspected of containing troponin complexes or components from damaged heart muscle is mixed with the antibody conjugate to form a reaction mixture and it is allowed to incubate. The reaction mixture is then applied to the aforementioned device. The sample flows through the membrane and the troponin complexes and components, bound to the antibody conjugate(s), bind to the respective immobilized antibodies in the discrete zones and excess, unbound antibody conjugate is washed away with a wash buffer. The signal is developed and read, either visually or instrumentally. In this assay procedure, the antibody conjugates bind to the free troponin I and T, to the

troponin I and T of the binary complexes and the troponin I or T of the ternary complex. The capture antibodies in discrete zones on the solid phase bind the antibody conjugates which are specific to the free troponin I and T and to the troponin complexes. This immunoassay allows quantification of the ternary troponin complex, the troponin T/C, I/T, I/C, oxidized I/T and oxidized I/C and the free troponin I (oxidized and reduced) and T. The inventive teachings described herein show that uncomplexed and complexed troponin exists in plasma and serum samples from patients with confirmed myocardial infarction. The determinations of the individual troponin ternary complex, the troponin I and T binary complexes and uncomplexed troponin I and T fractions may yield important clinical data relating to the type and extent of muscle damage, for example, from unstable angina or myocardial infarction or to the success of thrombolytic therapy.

A particularly preferred immunoassay for troponin measures the concentration of two or more forms of troponin, for example of free and complexed troponin I and or T, utilizing antibodies that have varying degrees of recognition for the different forms of troponin. An antibody or cocktail of antibodies are conjugated to a label or signal generator to form an antibody conjugate. The antibody conjugate has the ability to bind to each form of troponin that is to be quantified. A preferred antibody for the conjugate would be an "insensitive" antibody as defined above. Immobilized on a solid phase, for example, a membrane in a device, in discrete zones are antibodies or cocktails of antibodies that are complementary to the antibody conjugate antibody(s). The essential characteristics of the immobilized antibodies are defined in terms of their responses in the assay, and are,

therefore, discussed below after the assay is described. The essential features of the assay can be discussed for the case in which two forms of troponin are to be quantified. In the case when two forms, form 1 and 2, of troponin are to be quantified, two discrete zones are utilized. The system is calibrated using two sets of calibrators in a suitable matrix such as blood, plasma or serum. Preferably one set of calibrators contains form 1 of troponin at various concentrations and the other contains form 2 of troponin. Independently, each of the calibrators is mixed with the antibody conjugate to form a reaction mixture which is allowed to incubate. The reaction mixture is then applied to the aforementioned device. The sample flows through the membrane and the troponin, bound to the antibody conjugate(s), binds to the immobilized antibodies in the discrete zones and excess, unbound antibody conjugate is washed away with a wash buffer. The signal on both zones 1 and 2 is developed and measured for each calibrator. The simplest and preferred system would be one in which the assay signal is linear, or can be approximated to be linear, with respect to the troponin concentration. In this case, the calibration procedure would yield four independent assay slopes defined as follows:

$m_{11}$  = assay slope determined on zone 1 using form 1 of troponin as the calibrator (Eqn. 1a)

$m_{12}$  = assay slope determined on zone 1 using form 2 of troponin as the calibrator (Eqn. 1b)

$m_{21}$  = assay slope determined on zone 2 using form 1 of troponin as the calibrator (Eqn. 1c)



$m_{22}$  = assay slope determined on zone 2 using form 2  
of troponin as the calibrator (Eqn. 1d)

and two independent assay constants defined as follows:

5

$c_1$  = assay signal on zone 1 corresponding to zero  
troponin concentration. (Eqn. 2a)

10

$c_2$  = assay signal on zone 2 corresponding to zero  
troponin concentration. (Eqn. 2b)

15

One skilled in the art will recognize that the slopes and  
constants shown in Eqns. 1 and 2 could also be determined in  
a calibration in which the calibrator solutions are comprised  
of both forms of troponin at various different ratios of  
concentrations. A blood, plasma or serum sample suspected of  
containing forms 1 and 2 of troponin is then assayed as  
described above for the calibrators. The assay yields two  
signal values:  $S_1$  is the signal measured in zone 1 and  $S_2$  is  
the signal measured in zone 2. The two signals are described  
by two independent linear equations:

20

$$S_1 = m_{11}[\text{form 1}] + m_{12}[\text{form 2}] + c_1 \quad (\text{Eqn. 3a})$$

25

$$S_2 = m_{21}[\text{form 1}] + m_{22}[\text{form 2}] + c_2 \quad (\text{Eqn. 3b})$$

30

where [form 1] and [form 2] are the concentrations of form 1  
and 2 of troponin, respectively, in the sample. Equations 3  
can be solved using standard techniques of linear algebra to  
determine values for [form 1] and [form 2] in the sample if:

$$m_{11}m_{22} - m_{12}m_{21} \neq 0 \quad (\text{Eqn. 4})$$

(see for example, Mathematical Methods for Physicists, Acad. Press, NY, NY). Equations 1 and 4 define, therefore, the assay responses that are required for the antibodies in zone 1 and zone 2 to determine the concentrations of form 1 and form 2 of the troponin from the measured signals  $S_1$  and  $S_2$ . In general, the accuracy of the determination of the concentrations of form 1 and form 2 will increase with an increase in the difference shown in Eqn. 4. The value of the difference required to obtain a satisfactory result will depend on the precision of the calibration and the assay, and the accuracy required for the troponin concentration. For most assay systems, a preferred assay is one in which the antibody or cocktail of antibodies in at least one of the zones is sensitive to whether the troponin is form 1 or form 2, where "sensitive" is defined in a previous section. For example, either the ratio  $m_{11}/m_{12}$  or the ratio  $m_{22}/m_{21}$  or both should be larger than about 2. This procedure for determining the concentrations of two forms of troponin can be extended to more than two forms. If  $N$  forms are to be measured,  $N$  discrete zones must be used. A calibration must be performed utilizing a minimum of  $N+1$  calibrator solutions comprised of the  $N$  forms of troponin. A sample suspected of containing the  $N$  forms of troponin is assayed. The assay signal from each of the  $N$  zones is measured; the signal  $S_i$  from the  $i$ 'th zone can be expressed as follows:

$$S_i = \sum_{j=1}^{j=N} m_{ij} [\text{form } j] + c_i \quad (\text{Eqn. 5})$$

where  $m_{ij}$  is the assay slope determined on the  $i$ 'th zone using form  $j$  of the troponin as the calibrator and  $[\text{form } j]$  is the

concentration of form j of the troponin in the sample. Eqn 5 defines a set of N linear equations that can be solved to determine the concentrations of all N forms of troponin using standard techniques if the determinant of the matrix

5 comprised of the  $m_{ij}$ 's is not equal to zero. For most assay systems, a preferred assay is one in which at least N-1 of the zones contain an antibody or cocktail of antibodies that is sensitive to which form the troponin is in, i.e., the ratio  $m_{ii}/m_{ij}$ , where  $i \neq j$ , is greater than about 2. Finally,

10 these concepts can be extended to the case in which the assay response is not linear with the troponin concentration. In this case, the signal measured on the i'th of N zones will be given by the relation:

$$S_i = \sum_{j=1}^{j=N} F_{ij} \quad (\text{Eqn. 6})$$

where  $F_{ij}$  is a function of [form j] that describes the dose-response (Signal as a function of [form j]) of form j on zone

15 I and that is determined in a calibration procedure similar to those described above. Equation 6 describes a system of N non-linear equations which may be solved to determine the concentrations of the N forms of troponin using, for example,

20 approximation (computer) methods familiar to those skilled in the art.

In another embodiment of this invention, inhibitors which affect the affinity constants of the association of troponin I complexes or of troponin T complexes are added to

25 the sample prior to or with formation of the reaction mixture so that the free troponin I or troponin T is measured, respectively. The binding inhibitors may disrupt the troponin complexes or they may open up or partially unravel

the complex, such that some or all interactions between the troponin components are broken so that epitopes may be more easily assessable to the antibodies for binding. Inhibitors can be selected from the group of compounds which comprise, but is not limited to, metal chelating agents and peptides which compete with troponin I or troponin T for binding to proteins of the contractile apparatus. Metal chelating agents, particularly those which bind to calcium and magnesium, lower the affinity constant, for example, of troponin I and troponin C binding by about a factor of 10 as compared to the affinity in the presence of calcium (Biochemistry 33, 12729-12734 (1994)). Peptides which affect troponin I and troponin T binding to proteins of the contractile apparatus include mastoparan, melittin and peptide sequences which mimic the troponin I and T sequences at their binding domains with the proteins of the contractile apparatus ((Biochemistry 31, 11326-11334 (1992), J. Biol. Chem. 267, 15715-15723 (1992), Biochemistry 33, 8233-8239 (1994)). Other peptides which are useful as inhibitors are those which mimic the binding domains of the troponin components. The binding domains are described, for example, in Ann. Rev. Biophys. Biophys. Chem. 16, 535-559 (1987), and with the binding domain information, one skilled in the art synthesizes the peptide which mimics the peptide of the protein at the binding domain.

In another embodiment of this invention, troponin C is added to the sample prior to or with formation of the reaction mixture of the immunoassay so that all or nearly all of the troponin I or T in the sample will be bound by troponin C during the course of the assay. The troponin C concentration in the sample should be about 0.5  $\mu\text{g/ml}$  to 100  $\mu\text{g/ml}$  and preferably about 1 to 10  $\mu\text{g/ml}$ .

In another embodiment of this invention, troponin C and T are added to samples prior to or with formation of the reaction mixture of immunoassays for troponin I in order to bind all or nearly all of the troponin I in the form of the ternary troponin complex. The troponin C and T concentrations in the sample should be about 0.5 to 100  $\mu\text{g/ml}$  and preferably about 1 to 10  $\mu\text{g/ml}$ .

In yet another embodiment of this invention, troponin C and I are added to samples prior to or with formation of the reaction mixture of immunoassays for troponin T in order bind all or nearly all of the troponin T in the form of the ternary troponin complex. The troponin C and T concentrations in the sample should be about 0.5 to 100  $\mu\text{g/ml}$  and preferably about 1 to 10  $\mu\text{g/ml}$ .

These embodiments wherein troponin components are added to the sample prior to or with formation of the reaction mixture have several advantages.

Firstly, troponin I adsorbs tenaciously to glass surfaces and various membranes which can result in a lower measured troponin I concentration. Troponin T also adsorbs to surfaces. However, when bound to troponin C or in the ternary complex, the adsorptive characteristics of troponin I and T may be dramatically reduced. Thus, the recovery of the troponin I/C or T/C complex or the ternary complex can be better than troponin I or T. In this embodiment, antibodies that recognize the troponin I or T complexes are used in the immunoassays.

Secondly, if antibodies which bind only to the complexed troponin I or T are required, then the antibody selection process is less stringent because the antibodies are not required to have a similar affinity to the free troponin I or T.

One skilled in the art will appreciate the inventive teachings described herein and will recognize with these teachings that addition of reagents to a device or to each other, as recited in the embodiments, has many forms, and these forms are within the scope of this invention.

#### Stabilization of Troponin I or T for Calibrator Reagents

Troponin I and T are known to be unstable in aqueous formulations, as well as in patient samples. The (apparent) instabilities of the proteins, as taught herein, are related to the oxidation state of the troponin I, the propensity of troponin I and T to form complexes with other troponin proteins and the adsorptive characteristics of troponin I and T onto surfaces.

Stabilization of troponin I is performed by the intramolecular oxidation of the cysteines and the protein is stored without thiol reducing agents, such as mercaptoethanol, dithiothreitol and the like.

The storage of troponin I in solutions containing high concentrations of thiol reductants will maintain the cysteines, overall, in the reduced form. However, intramolecular oxidation and reduction of a protein is a dynamic process whereby the protein will exist for some time in the oxidized form even in the presence of the reductants. In the case of reductants, such as mercaptoethanol or N-acetylcysteine, that is, reductant molecules with only a single thiol group, mixed disulfides of the reductant and the protein thiol will form. The half-life of this mixed disulfide will be a function of the reductant concentration and the rate of intramolecular oxidation; that is, the mixed disulfide can be reduced by both the thiol reductant reagent and the other protein cysteine, assuming that both cysteines

are not in the mixed disulfide form. In the case of reducing the intramolecularly oxidized troponin I, the reductant with a single thiol group will reduce the intramolecular cystine to yield a cysteine and a mixed disulfide of the protein.

5 The mixed disulfide of the protein will be reduced by either the cysteine of the protein or the thiol reductant. This process continues and eventually the reductant concentration is depleted to a level where it can no longer maintain the protein in the reduced state. As the reductant concentration  
10 approaches the concentration of thiol in the troponin I, the protein cysteine and the thiol reductant reagent can form mixed disulfides, which will not be reduced by the thiol reductant. Alternatively, the protein will oxidize, intramolecularly, and the thiol reductant is not in  
15 sufficient concentration to reduce the cystine. The end result, upon depletion of the thiol reductant, will be a mixture of troponin I which is in the intramolecularly oxidized form and protein which is in the mixed disulfide form. Each of these forms of troponin I has a different  
20 conformation.

In the case of utilizing thiol reductant reagents which possess two thiol groups, for example dithiothreitol or dithioerythritol, the end result, upon depletion of the thiol reductant, will be only the oxidized form of the troponin I.

25 Therefore, antibodies which are sensitive to the oxidation state of the troponin I will differentially recognize the various forms of the troponin I in the immunoassay. The immunoassay will then measure an inaccurate concentration of troponin I.

30 A preferred composition of stabilized troponin I comprises an aqueous solution of the intramolecularly oxidized troponin I. A particularly preferred composition

of stabilized troponin I and T comprises a buffered solution of the ternary complex of troponin I, T and C in the presence or absence of calcium and magnesium salts. A preferred range of pH of the solution is between 6 and 9 and a range of calcium and magnesium salts concentrations, for example calcium and magnesium chloride, of between 0.01 mM and 10 mM. A particularly preferred buffered solution consists of up to about 100% human serum or plasma. The ternary complex can be formed from the component troponin I, T and C, or alternatively, it can be isolated and purified from cardiac or skeletal muscle (Methods Enzymol. 85, 241-263 (1982)).

#### Methods for Improving the Recovery of Troponin I in Membranes

The adsorption of troponin I and T to surfaces and to various proteins is known to occur and this phenomenon can lower the measured troponin concentration. In particular, when immunoassays are performed in devices or instruments which have a large surface area, for example, when membranes or latex particles are incorporated into the assay process, the surface area which is exposed to the sample can lower the recovery of troponin. Membranes made up of nylon or compositions of glass fibers having sizes of between 2 mm x 2 mm x 1 mm and 40 mm x 40 mm x 5 mm can influence the recovery of troponin I and T when coupled with the assay process.

The troponin I and T molecules have a high degree of basic amino acids. At physiological pH, the basic amino acids are largely positively charged and these charged groups contribute to the adsorptive behavior of the proteins.

In a preferred embodiment of this invention, various components are added to membranes or latex particles to improve the recovery of troponin I and T in the immunoassay process. Specifically, peptides or proteins



which are also strongly basic are added to membranes or latex particles or surfaces of devices involved in the assay process prior to or with addition of the sample or reaction mixture. Preferred compounds for this use include peptides, proteins and polymers with pI values greater than about 8. Included in this group are salmine, lysozyme, cytochromes, protamine, polylysine, polyvinyl amine, melittin and mastoparan. Concentrations of blocking reagents which are added to surfaces or membranes range from about 0.01 mg/ml to 100 mg/ml and typically about 0.1 mg/ml to 10 mg/ml.

### Experimental Section

#### Example 1

#### Preparation of Reagents for Troponin ELISA Immunoassays

#### Preparation of Anti-Troponin Antibody Alkaline Phosphatase Conjugates

Alkaline phosphatase (Calzyme, San Luis Obispo, CA) in 50 mM potassium phosphate, 10 mM potassium borate, 150 mM sodium chloride, pH 7.0, at 10 mg/ml was derivatized with SMCC (succinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate, Pierce Chemical Co., Rockford, IL) at a 15/1 molar ratio of SMCC/enzyme. The derivatization was performed at room temperature for 90 min and subsequently chromatographed on a GH-25 column (Amicon Corp., Beverly, MA) equilibrated in 50 mM potassium phosphate, 10 mM potassium borate, 150 mM sodium chloride, pH 7.0.

The anti-troponin antibodies in 50 mM potassium phosphate, 10 mM potassium borate, 150 mM sodium chloride, pH 7.0, at 10 mg/ml were derivatized with SPDP (N-succinimidyl-3-[2-pyridyldithiol]propionate, Pierce Chemical Co.) at a 10/1 molar ratio of SPDP/antibody. The antibody was diluted to 2 mg/ml and Dithiothreitol and taurine were added to the

solution at final concentrations of 1 mM and 20 mM, respectively, and the solution was subsequently incubated at room temperature for 30 min. The antibody-SPDP was chromatographed on a GH-25 column (Amicon Corp.) equilibrated in 50 mM potassium phosphate, 10 mM potassium borate, 150 mM sodium chloride, 0.1 mM ethylenediamine tetraacetic acid, pH 7.0.

The SMCC-alkaline phosphatase (in 50 mM potassium phosphate, 10 mM potassium borate, 150 mM sodium chloride, 5 mM magnesium chloride, pH 7.0) and the thiol-antibody (in 50 mM potassium phosphate, 10 mM potassium borate, 150 mM sodium chloride, 0.1 mM ethylenediamine tetraacetic acid pH 7.0), both diluted to 1 mg/ml, were rapidly added to each other with mixing in equimolar amounts. The solution was incubated at room temperature for 3 hours, after which N-ethyl maleimide was added to a final concentration of 2 mM.

#### Preparation of Biotinylated Troponin Antibodies

Biotin-XX, succinimidyl ester (6-((6-((biotinoyl)amino)hexanoyl)amino)hexanoic acid, succinimidyl ester, Molecular Probes, Eugene, OR) at 40 mM in dimethylformamide was added slowly with mixing to an antibody solution at 2 mg/ml in 50 mM potassium borate, 150 mM sodium chloride, pH 8.2, (BBS) to achieve a final molar ratio of 20/1 biotin-XX/antibody. The solution was incubated at room temperature for 2 h, after which the solution was dialyzed at 4°C for at least 12 h.

#### Preparation of Avidin-HS Magnetic Latex

One ml of Estapor Paramagnetic latex particles (0.94  $\mu$ , Bangs Laboratories, Carmel, IN, at 10% solids, washed 4 times with deionized water) in water was added to 9

ml of 0.55 mg/ml avidin-HS (Scripps Laboratories, San Diego, CA) in 50 mM Tris hydrochloride, 150 mM sodium chloride, pH 7.5. The latex solution was incubated at 45 C for 2 h. The latex was washed 3 times, each with 10 ml BBS, and resuspended in 10 ml BBS.

## Example 2

### Immunoassay of Human cardiac Troponin I and Troponin T

Two immunoassay protocols are described. They were used to detect Troponin I and Troponin T, present in human serum, plasma or in solutions containing purified proteins.

#### Protocol A

The sample containing troponin I or troponin T was diluted to 1-10 ng/ml troponin I or troponin T in an assay buffer (hereafter called assay buffer) containing 10 mM 3-(N-morpholino) propane sulfonic acid, 650 mM sodium chloride, 1 mM magnesium chloride, 0.1 mM zinc chloride, 1 mg/ml polvinyl alcohol (10,000 m.w.), 10 mg/ml bovine serum albumin, 1 mg/ml sodium azide, pH 7.0. To 25  $\mu$ l of diluted sample in a microtiter plate well was added 50  $\mu$ l of assay buffer containing 2.5  $\mu$ g/ml anti-troponin I or troponin T antibody conjugates (Example 1) and 2.5  $\mu$ g/ml biotinylated anti-troponin I or troponin T polyclonal antibody (Example 1) to form a reaction mixture. After a 30 minute incubation of the reaction mixture at room temperature, 25  $\mu$ l of avidin-HS coated magnetic latex (Example 1; 0.5% latex in assay buffer) was added to the microtiter plate well, followed by a 5 minute incubation at room temperature. The magnetic latex was pelleted using a microtiter plate magnet (Perceptive Diagnostics, Cambridge, MA) and washed twice in BBS-Tween (20 mM borate, 150 mM sodium chloride, 0.1 mg/ml sodium azide,

0.02% Polyoxyethylene-20-Sorbitan Monolaurate (Tween-20), pH 8.2) and once in TBS (40 mM Tris, 150 mM sodium chloride, pH 7.5) The pellet was resuspended in ELISA amplification reagents (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. After the amplification was complete, the magnetic latex was pelleted and 80  $\mu$ l of the colored supernatant was transferred to a fresh microtiter plate. The absorbance at 490 nm ( $OD_{490}$ ) was measured using a microtiter plate reader (Molecular Devices, Palo Alto, CA).

#### Protocol B

The sample containing troponin I or troponin T was diluted into assay buffer as described in protocol A. To 80  $\mu$ l of diluted sample in a microtiter plate well was added 40  $\mu$ l of assay buffer also containing 30  $\mu$ g/ml anti-troponin I or troponin T monoclonal antibody and 7.5  $\mu$ g/ml biotinylated anti-troponin I or troponin T polyclonal antibody (Example 1) that was complimentary to the monoclonal antibody to form the reaction mixture. Aliquots ( 25  $\mu$ l) were removed at various times (2 minutes to 24 hours) and were added to microtiter plate wells containing 25  $\mu$ l of avidin-HS coated magnetic latex (0.5% latex solids in assay buffer), followed by a 5 minute incubation. The magnetic latex was pelleted and washed once in BBS-Tween and once in assay buffer. The pellet was resuspended in 25  $\mu$ l of assay buffer also containing 5  $\mu$ g/ml of goat anti mouse kappa antibody conjugated to alkaline phosphatase (Southern BioTechnology Associates, Inc., Birmingham, AL) followed by a 15 minute incubation. The magnetic latex was pelleted and the remainder of the assay was performed as indicated in Protocol A.

Example 3Selection of Anti-Troponin I Antibodies that Bind the Oxidized, the Reduced or Both the Oxidized and Reduced Forms of Human Cardiac Troponin I

5                   Anti-troponin I antibody conjugates (Example 1) and complimentary biotinylated troponin I polyclonal antibodies (Example 1) were tested for recognition of intramolecularly oxidized or reduced troponin I. The anti troponin I monoclonal antibodies tested were: clone 2D5 and clone 1A12  
10                   (BiosPacific, Emeryville, CA), clone 110 and 111 (Research Diagnostics, Inc., Flanders, N.J. ) and clone TRI-7 F81 (DAKO Corporation, Carpinteria, CA). The biotinylated anti-troponin I antibodies tested were affinity-purified goat polyclonals, specified as peptide 1, peptide 2, peptide 3 or  
15                   peptide 4 specific (BiosPacific, Emeryville, CA). Human cardiac troponin I, (P. Cummins, University of Birmingham, Birmingham, UK) was air oxidized at 1.0  $\mu\text{g/ml}$  as described in example 4 to form the intramolecular disulfide. The oxidized troponin I was diluted to 1-10 ng/ml in assay buffer either  
20                   without (oxidized sample) or with (reduced sample) dithiothreitol (DTT) at a final concentration of 3mM, followed by a 3 hour incubation at room temperature to allow reduction of the disulfide by DTT. The oxidized and reduced samples were assayed without further dilution using Protocol  
25                   A of Example 2. The results are shown in Table 1 and are expressed in terms of a ratio of the assay slope for oxidized troponin I (TnI) divided by the assay slope for reduced troponin I. The assay slope increases with increasing recognition of troponin I by the antibody pair.

30                   The data show that antibodies can be selected that either preferentially bind oxidized or reduced troponin I or bind oxidized and reduced troponin I approximately the same.

Selection of the antibodies without regard to the oxidation-reduction state of troponin I can lead to a substantial error in the quantification of the troponin I concentration.

66-0320-10-6-1-50

Table 1

Anti troponin I monoclonal antibody	Anti troponin I polyclonal antibody	Ratio of assay slopes (oxized TnI/reduced TnI)
Clone 2D5	peptide 1 specific	8.3
Clone 2D5	peptide 3 specific	10
Clone 1A12	peptide 1 specific	0.6
Clone 1A12	peptide 3 specific	1.3
Clone 1A12	peptide 4 specific	1.2
Clone TRI-7 F81	peptide 1 specific	0.5
Clone TRI-7 F81	peptide 2 specific	0.5
Clone TRI-7 F81	peptide 3 specific	0.5
Clone TRI-7 F81	peptide 4 specific	0.5
Clone 110	peptide 4 specific	0.8
Clone 111	peptide 3 specific	1.0
Clone 111	peptide 4 specific	0.4

Example 4Oxidation-Reduction of Purified Human Cardiac Troponin I

5                   The kinetics of intramolecular oxidation and  
reduction of purified troponin I (P. Cummins, University of  
Birmingham, UK) was measured with an immunoassay (Protocol A,  
Example 2) using a clone 2D5 antibody conjugate (Example 1)  
and biotinylated goat anti troponin I peptide 1 polyclonal  
10 antibody (Example 1). This antibody pair binds strongly to  
oxidized troponin I and weakly to reduced troponin I as  
described in Example 3. The results of the assay are  
expressed in terms of an assay slope [OD<sub>490</sub> per ng/ml total  
(oxidized + reduced) troponin I] in the linear range of the  
15 assay. The assay slope increases with the fraction of  
oxidized troponin I.

Air Oxidation of Reduced Troponin I

                  The rate of air oxidation of troponin I at two  
troponin I concentrations was measured. Reduced troponin I at  
20 0.27 mg/ml in a buffer containing 20 mM Tris-HCl, 0.5 M  
sodium chloride, 60 mM 2-mercaptoethanol, pH 7.5, was diluted  
to either 1300 ng/ml or 10 ng/ml in assay buffer containing  
either no or 25 mM 2-mercaptoethanol. The solutions were  
incubated at room temperature. Aliquots were taken after  
25 various incubation times, as indicated in Figure 1a, diluted  
to 4 and 8 ng/ml troponin I in assay buffer, and assayed  
immediately. The results are shown in Figure 1a, wherein the  
error bars represent 1 standard deviation (SD).

Peroxide Oxidation of Reduced Troponin I

30                   Peroxide (Fisher, unstabilized) was added (final  
concentration of peroxide 2 mM or 20 mM) to an aliquot of the  
1300 ng/ml solution of reduced troponin I (see this example,



air oxidation) immediately after the troponin I was diluted from the 0.27 mg/ml stock solution. The solutions were incubated at room temperature. Aliquots were taken after various incubation times, as indicated in Figure 1b, treated with catalase (Calbiochem, La Jolla, CA; 0.01 mg/ml final concentration for 5 minutes) to remove the peroxide, diluted to 4 and 8 ng/ml troponin I, and assayed immediately. The results are shown in Figure 1b, wherein the error bars represent 1 SD.

#### DTT Reduction of Oxidized Troponin I

Troponin I that was incubated (air oxidized) at 1000 ng/ml in assay buffer for 15 hours at room temperature was diluted to 4 and 8 ng/ml in assay buffer. DTT was added to a final concentration of 0, 1.5 and 3.0 mM followed by incubation at room temperature for the times indicated in Figure 2. The aliquots were then assayed for troponin I. After steady state was reached (approximately 6 hours), aliquots (100  $\mu$ l) from the three DTT concentration samples were reoxidized with 20 mM peroxide for 15 minutes, treated with catalase for 5 minutes and assayed. The results are shown in Figure 2, wherein the error bars represent 1 SD.

The data show that the results of an immunoassay can vary over time if the oxidation-reduction state of the troponin I is allowed to change. The oxidation-reduction state of troponin I, and thus the immunoassay results, can be reversibly changed and greatly stabilized over time by the use of oxidants and reductants.

#### Example 5

#### Alkylation of Reduced Troponin I

Troponin I was rapidly alkylated using various alkylating reagents. The stock reduced troponin I

(University of Birmingham) was at 0.27 mg/ml in 20 mM Tris hydrochloride, 0.5 M sodium chloride, 50 mM 2-mercaptoethanol. Three alkylation reactions (#1-3) were performed and a control was prepared (#4):

- 5      1. 20  $\mu$ l of stock troponin I was added to 20  $\mu$ l 0.5 M potassium borate, 0.2 mM ethylenediamine tetraacetic acid, pH 8.0 and subsequently, 10  $\mu$ l 398 mM iodoacetamide was added.
2. 20  $\mu$ l of stock troponin I was added to 20  $\mu$ l 0.5 M potassium borate, 0.2 mM ethylenediamine tetraacetic acid, pH  
10      8.0 and subsequently, 10  $\mu$ l 398 mM iodoacetic acid was added.
3. 20  $\mu$ l of stock troponin I was added to 20  $\mu$ l 0.5 M potassium borate, 0.2 mM ethylenediamine tetraacetic acid, pH 8.0 and subsequently, 12.5  $\mu$ l 319 mM N-ethylmaleimide was added.
- 15      4. 20  $\mu$ l of stock troponin I was added to 20  $\mu$ l 0.5 M potassium borate, 0.2 mM ethylenediamine tetraacetic acid, pH 8.0 and subsequently, 10  $\mu$ l 0.5 M potassium borate, 0.2 mM ethylenediamine tetraacetic acid, pH 8.0 was added.

The reactions were incubated at room temperature  
20      for 1 h 25 min. During this incubation, the stock troponin I was kept on ice. Aliquots (24  $\mu$ l) of each solution (1-4) were added to 0.9  $\mu$ l of 2.9 M mercaptoethanol and were incubated at room temperature for 15 min, after which the samples were frozen in liquid nitrogen. The remaining  
25      aliquots of each solution (1-4) were also frozen in liquid nitrogen with no further treatment.

#### Example 6

##### Immunoassay of Alkylated Troponin I

30      Freshly thawed Troponin I alkylated (Example 5) with N-ethyl maleimide (NEM), iodoacetic acid (IHAC), iodoacetamide (IAM), or not alkylated (control sample,

Example 5) was diluted to 1-10 ng/ml in assay buffer. A freshly thawed aliquot of the reduced stock troponin I (Example 5) was diluted (standard sample) into assay buffer containing either 0 or 3 mM DTT. Aliquots (25  $\mu$ l) of all dilutions were taken after either a 0.5 hour or 5.5 hour incubation at room temperature and assayed (Protocol A, Example 2) using a clone 2D5 anti troponin I monoclonal antibody conjugate and biotinylated goat anti troponin I peptide 1 specific polyclonal antibody (Example 1). This antibody pair binds strongly to oxidized troponin I and weakly to reduced troponin I (Examples 3 and 4). Troponin I will remain substantially reduced during a 0.5 hour incubation but will be almost completely oxidized (by air) after a 5.5 hour incubation unless DTT is present to stabilize the reduced form (see Example 4). The results are shown in Table 2 and are given in terms of assay slope (OD<sub>490</sub> per ng/ml total troponin I) in the linear range. A larger assay slope indicates a stronger binding interaction between the antibodies and the troponin I.

The data show that the antibody pair binds alkylated troponin I similarly to reduced troponin I, that is, weakly in comparison with oxidized troponin I. Furthermore, alkylation stabilizes the immunoassay result with respect to time, similarly to the effect observed by the use of oxidants or reductants to stabilize the oxidation-reduction state of troponin I (Example 4). The lower and more stable assay slope of the control sample as compared with the standard sample is explained by the presence of mixed disulfides formed between the two cysteine residues of the control sample troponin I and 2-mercaptoethanol during the room temperature incubation of the control sample at pH 8 (see Example 5).

Table 2

Sample	Assay slope (0.5 hour incubation)	Assay slope (5.5 hour incubation)	Ratio of assay slopes (5.5 hour/0.5 hour)
reduced TnI standard (+DTT)	0.030	0.030	1.0
reduced TnI standard (-DTT)	0.058	0.21	3.6
TnI Control	0.037	0.078	2.1
TnI alkylated with NEM	0.023	0.026	1.1
TnI alkylated with IAM	0.013	0.013	1.0
TnI alkylated with IHAC	$\leq 0.01$	$\leq 0.01$	

Example 7Effect of Peroxide on Immunoassay of Cardiac Troponin I from Patients with Confirmed Myocardial Infarction

Frozen Human serum or plasma, drawn in heparin tubes from patients with confirmed myocardial infarction, was obtained from local hospitals. The serum or plasma was thawed at room temperature and immediately split into two aliquots. One aliquot was oxidized at room temperature by the addition of peroxide at a final concentration of 20 mM. The second aliquot was untreated. The oxidation reaction was stopped after 20 minutes by the addition of catalase at a final concentration of 0.01 mg/ml. Ten minutes after the catalase was added both the oxidized and the untreated aliquots were

diluted serially by factors of four in assay buffer and assayed immediately for cardiac troponin I using the 2D5 anti troponin I conjugate and biotinylated anti troponin I peptide 3 specific antibodies (Example 2, Protocol A). This  
5 complimentary antibody pair binds oxidized troponin I strongly and reduced troponin I weakly (Example 3). Air oxidized (example 4) purified troponin I (P. Cummins, University of Birmingham), diluted to 2, 4, and 8 ng/ml in assay buffer, was assayed with the same antibody reagents to  
10 construct a standard curve. The concentration of troponin I in the neat oxidized or untreated serum or plasma sample (Table 3) was calculated from this standard curve using the OD<sub>490</sub> measurements that fell within the linear range of the assay.

15           The data show that oxidation of serum or plasma samples from patients with confirmed myocardial infarction can have a substantial effect on the concentration of cardiac troponin I determined by immunoassay. Immunoassay of troponin I in serum or plasma without regard to the oxidation state of  
20 the troponin I could lead to a serious underestimation of the troponin I concentration and result in the non-diagnosis of a myocardial infarction.

Table 3

Sample	Time between sample collection and freezing (hours)	Troponin I concentration by assay of untreated sample (ng/ml)	Troponin I concentration by assay of Peroxide oxidized sample (ng/ml)
1 Plasma	2	6.3	9.4
2 Plasma	6.5	0.8	1.0
3 Serum	9.3	6.6	8.3
4 Serum	6.5	31.9	46.5
5 Plasma	6.5	31.7	49.7
6 Plasma	9.5	0.6	1.0
7 Serum	11.5	0.4	0.4
8 Plasma	5.0	4.5	5.4
9 Serum	10.5	1.6	2.3
10 Plasma or Serum	unknown	13.6	13.2

Example 8

Effect of Peroxide on Immunoassay of Cardiac Troponin I in Human Plasma after two Freeze/Thaw Cycles.

Plasma sample number 5 (Table 3, Example 7) was stored untreated on ice for three hours after it was initially thawed and then refrozen and stored at -70 C for several days. The plasma was thawed at room temperature and split into two aliquots; one was oxidized with peroxide and the other was left untreated as described in Example 7. The concentration of troponin I in the oxidized and untreated aliquots was determined immediately by the immunoassay described in example 7 and was found to be 53.9 ng/ml in the untreated aliquot and 56.4 ng/ml in the oxidized aliquot.

The data show that oxidation of the plasma after the second thaw did not have a substantial effect on the concentration of cardiac troponin I determined by immunoassay.

5

Example 9

Immunoassay of Cardiac Troponin I in Oxidized and Reduced Plasma From a Patient With Myocardial Infarction

10 Frozen Human plasma drawn in heparin tubes from a patient with a confirmed myocardial infarction was obtained from a local hospital. The plasma was thawed at room temperature and immediately split into two aliquots. One aliquot was oxidized with peroxide as described in Example 7. The other aliquot was reduced by addition of DTT to a final  
15 concentration of 10mM, followed by a 3 hour incubation at room temperature. The oxidized aliquot was then diluted serially by factors of 2 into assay buffer and the reduced aliquot was diluted serially by factors of 2 into assay buffer containing 3 mM DTT. The diluted aliquots were assayed  
20 for troponin I immediately (Protocol A, Example 2) either with the complementary antibody pair clone 2D5 anti troponin I conjugate and biotinylated anti troponin I peptide 3 polyclonal antibody or with the complementary antibody pair clone TRI-7 F81 anti troponin I conjugate and biotinylated  
25 anti troponin I peptide 3 polyclonal antibody. Purified troponin I (University of Birmingham) which was air oxidized (example 4) was diluted to 2, 4, and 8 ng/ml in assay buffer and was used to construct the standard curve from which the concentration of troponin I in the neat oxidized or reduced  
30 plasma sample was determined. The results are shown in Table 4.

The data show that chemical oxidation and reduction of cardiac troponin I in the plasma sample affects the recognition of the tested antibody pairs for the troponin I in a manner similar to that observed for purified troponin I (Example 3).

Table 4

Monoclonal antibody conjugate	Assayed troponin I concentration (ng/ml) in oxidized plasma	Assayed troponin I concentration (ng/ml) in reduced plasma	Ratio of troponin I concentrations (oxidized plasma/reduced plasma)
Clone 2D5	82	< 1	> 82
Clone TRI-7 F81	52.8	77.5	0.68

Example 10

Selection of Anti Troponin I Antibodies That Are Either Sensitive or Insensitive To The Binding of Troponin C to Troponin I

Monoclonal anti troponin I conjugates and complimentary biotinylated anti troponin I polyclonal antibodies (Example 1) were tested for their recognition of free troponin I and troponin I bound to troponin C in a binary complex. Four types of troponin I samples were prepared at room temperature and assayed for troponin I; they are: oxidized troponin I with and without added troponin C and reduced troponin I with and without added troponin C. Oxidized (by air, see Example 4) Human cardiac troponin I (P. Cummins, University of Birmingham) was diluted to 2, 4, and 8



ng/ml in assay buffer containing 2 mM calcium chloride. One aliquot of each concentration of troponin I was either untreated or reduced by the addition of DTT to a final concentration of 3 mM from a 30 mM DTT stock solution in assay buffer to form a reduction reaction. Three hours after the reduction reaction was started, each oxidized and reduced troponin I aliquot was split into two aliquots; to one aliquot was added human cardiac troponin C (Bio-Tech International Inc., Seattle, WA) to a final concentration of 0.1 mg/ml from a 1 mg/ml stock solution in 20 mM potassium phosphate, 4 mM potassium borate, 150 mM sodium chloride, pH 7.0 to form a binding reaction mixture, and to the other aliquot was added the same volume of the above buffer without troponin C. One hour after the troponin C was added, all the aliquots were assayed for troponin I (Protocol A, Example 2) using the antibody pairs listed in Table 5. The results in Table 5 are expressed as a fractional assay response which was determined by dividing the assay slope in the presence of troponin C by the assay slope in the absence of troponin C.

The results in Table 5 show that some antibody pairs recognize free troponin I and troponin I bound to troponin C equally well, while other antibody pairs recognize only free troponin I. An immunoassay with antibodies that do not recognize troponin I bound to troponin C will underestimate the total troponin I concentration when some of the troponin I is present as the troponin I/C binary complex.

Table 5

Anti troponin I antibody conjugate	Biotinylated anti troponin I polyclonal antibody	Fractional assay response	
		Oxidized troponin I	Reduced troponin I
Clone 2D5	Peptide 3 specific	0.81	0.60
Clone 111	Peptide 1 specific	0.83	Not determined
Clone 111	Peptide 3 specific	0.47	0.52
Clone 111	Peptide 4 specific	0.59	0.19
Clone 110	Peptide 4 specific	0.96	0.48
Clone 1A12	Peptide 1 specific	< 0.05	< 0.05
Clone 1A12	Peptide 3 specific	< 0.05	< 0.05
Clone 1A12	Peptide 4 specific	< 0.05	< 0.05
Clone TR7 F81	Peptide 1 specific	0.74	0.79
Clone TR7 F81	Peptide 2 specific	0.92	1.04
Clone TR7 F81	Peptide 3 specific	0.94	0.97
Clone TR7 F81	Peptide 4 specific	0.70	0.79

Example 11

Effect of Troponin T, EDTA, Melittin and Mastoparan on a Troponin I Immunoassay With Troponin C Present in large Excess Over Troponin I

5                    Ethylenediamine tetraacetic acid (EDTA) lowers the binding affinity of troponin I for troponin T and troponin C by chelating calcium and magnesium ions. Melittin lowers the affinity of troponin I for troponin C by binding to troponin C. The effectiveness of EDTA and Melittin (hereafter referred  
10 to as binding inhibitors) in breaking up the binary complex of troponin I and troponin C in the presence and absence of troponin T was assessed. Oxidized Human cardiac troponin I (P. Cummins, University of Birmingham) at 1.0 ug/ml in assay buffer containing 2 mM calcium chloride was reduced with  
15 dithiothreitol at a final concentration of 3 mM for three hours at room temperature. The reduced troponin I was diluted to 2 and 4 ng/ml in assay buffer containing 2 mM calcium chloride and 3 mM dithiothreitol. Each concentration was split into four aliquots to which were added human cardiac  
20 troponin C (Bio-tech International, Inc.) to final concentrations of 0, 0.1, 1.0, and 10.0  $\mu$ g/ml from 100-fold excess stock solutions in 20 mM potassium phosphate, 4 mM potassium borate, 150 mM sodium chloride, pH 7.0. Each of the resulting aliquots were further split into two aliquots to  
25 which was added human troponin T (Scripps Labs) to a final concentration of either 0.0 or 0.1  $\mu$ g/ml from a 100-fold excess stock solution in deionized water. The aliquots were incubated at room temperature for one hour after the addition of troponin T, then assayed for troponin I (Protocol B,  
30 Example 2). The antibody solution added to the microtiter plate wells contained 30  $\mu$ g/ml clone 1A12 anti troponin I and 7.5  $\mu$ g/ml biotinylated anti troponin I peptide 4 specific

antibodies (example 1) either without or with binding inhibitors (30 mM EDTA and 0.15 mM Melittin (Sigma Chemical, Co., St. Louis, MO)). Aliquots of the reaction mixtures formed by the addition of antibodies to the troponin I samples were removed after 0.5 h and were further treated as described in Protocol B, Example 2. The assay results for the samples containing no troponin C or T and no binding inhibitors were used to construct a standard dose-response curve. The effect on the standard curve of addition of the binding inhibitors to the assay was tested and found to be negligible. The fractional assay response (shown in Figure 3) for samples containing inhibitors and troponin components was determined by dividing the assay slope for each sample by the slope of the standard curve.

The data show that in the presence of troponin C, the troponin I concentration is largely underestimated. The binding inhibitors almost completely reverse the effect of troponin C. The presence of troponin T in the absence of troponin C has no effect on the troponin I immunoassay. (Data not shown in Figure 3). In the presence of troponin C and T, the measured concentration of troponin I is dramatically reduced. The binding inhibitors appear to be less effective at opening up or partially unraveling the troponin complex when the complex is ternary than when the complex is binary. Mastoparan or Melittin at 0.1 mM was also tested as a binding inhibitor to dissociate the I/C complex for a troponin C concentration of 10 ug/ml (Data not shown). The melittin was as effective as the melittin/EDTA (Figure 3) at increasing the fractional assay response, while the mastoparan was about one third as effective as the melittin at the concentrations tested.

Example 12

Effect of Binding Inhibitors on an Immunoassay of Troponin I  
in the Presence of Troponin C or Troponin C and T

Solutions containing 1.0  $\mu\text{g/ml}$  purified human  
5 cardiac troponin I (reduced by DTT, Example 4) and either 1.2  
 $\mu\text{g/ml}$  human cardiac troponin C (Bio-tech International, Inc.)  
or 1.2  $\mu\text{g/ml}$  troponin C and 3.1  $\mu\text{g/ml}$  human cardiac troponin  
T (Scripps Labs) were incubated for 2 hours at room  
temperature in assay buffer containing 3 mM DTT. Troponin C  
10 was added to troponin I prior to addition of troponin T. The  
troponin solutions were diluted to 2, 4 and 8 ng/ml in terms  
of troponin I concentration in assay buffer containing 2 mM  
calcium chloride and 0.5 mM DTT and assayed immediately with  
and without binding inhibitors as described in example 11.  
15 Aliquots of the reaction mixtures of antibodies and troponin  
components were removed 0.5 h and 2.2 h after the antibodies  
were added. These aliquots were further treated as described  
in Protocol B, Example 2. Reduced troponin I without added  
troponin C and T and without binding inhibitors was assayed  
20 to produce a standard curve. The effect on the standard  
curve of addition of the binding inhibitors to the assay was  
tested and found to be negligible. The results are expressed  
in Table 6 as a fraction assay response which was determined  
by dividing the assay slope for each sample by the slope of  
25 the standard curve.

The data show that troponin T and C present in  
approximately a two fold molar excess above the troponin I  
concentration substantially lowers the amount of troponin I  
measured in the immunoassay. Troponin C alone has a smaller  
30 effect on the measured troponin I concentration at the  
antibody concentrations used in this assay. The binding

inhibitors partially reverse the effect of troponin C and T at 0.5 h incubation and completely at 2.2 h.

Table 6

Time after antibodies added (hours)	Fractional assay response			
	With troponin C		With troponin C and T	
	without binding inhibitors	with binding inhibitors	without binding inhibitors	with binding inhibitors
0.5	0.88	0.94	0.55	0.75
2.2	1.15	1.02	0.44	1.02

### Example 13

#### Assay of Purified Human Cardiac Ternary Troponin Complex for Troponin I

Purified human cardiac ternary troponin complex (Bio-tech International, Inc., 3 mg/ml stock solution in a buffer containing 20 mM sodium citrate, pH6) was diluted to concentrations ranging from 1 to 15 ng/ml total troponin I in assay buffer containing 2 mM calcium chloride but without the 0.1 mM zinc chloride or the 1 mM magnesium chloride (hereafter called metal-free assay buffer). The diluted solutions were assayed for troponin I with and without binding inhibitors present as described in Example 11. Aliquots of the reaction mixtures of the antibodies with the troponin complex were taken at the times indicated in Figure 4 and were further treated as described in Protocol B,

Example 2. Purified troponin I (Bio-tech International, Inc.) was assayed and used to construct a standard curve. The effect on the standard curve of addition of the binding inhibitors to the assay was tested and found to be negligible. The fractional assay response shown in Figure 4 was determined by dividing the assay slope for the complex (OD<sub>490</sub> as a function of total troponin I concentration) by the slope of the standard curve.

The results show that troponin I that is bound in the ternary complex with troponin C and T is not recognized very well by the antibodies at the antibody and troponin I concentrations used in this assay, even after a very long incubation time. In particular, the 30 minute time point had no detectable troponin I, with or without inhibitors. The binding inhibitors slowly open up or partially unravel the complex and thus slowly increase the fraction of troponin I recognized by the antibodies.

#### Example 14

#### Effect of Binding Inhibitors on a Troponin I Immunoassay of Plasma and Serum from Patients with Confirmed Myocardial Infarction

Frozen serum and plasma drawn in EDTA and Heparin tubes from patients with a confirmed myocardial infarction were obtained from local hospitals and thawed at room temperature. Calcium chloride was added to a final concentration of 6 mM (to bind all the EDTA) and the resultant solution was incubated for two hours at room temperature and then overnight at 4 C. The incubated samples were diluted by factors of two to a maximum dilution factor of 256 in metal-free assay buffer. The diluted samples were immediately assayed for troponin I with and without binding inhibitors as

described in Example 11, with the exception that the polyclonal antibody was goat anti-troponin I peptide 3 specific. Aliquots of the reaction mixture formed by the addition of antibodies to the diluted samples were taken at the times indicated in Figures 5a-f and further treated as described in Example 2, Protocol B. Oxidized troponin I (University of Birmingham) at 2,4 and 8 ng/ml in metal-free assay buffer was assayed to produce a standard curve. The effect on the standard curve of addition of the binding inhibitors to the assay was tested and found to be negligible. The OD<sub>490</sub> values measured for the diluted serum or plasma samples were plotted as a function of the inverse of the dilution factor. The slope in the linear region of the resultant curve (typically at OD<sub>490</sub> < 2, which corresponds to a troponin I concentration of less than 8 ng/ml) was divided by the slope of the standard curve to obtain the concentration of troponin I in the neat serum or plasma sample shown in Figures 5a-f. Each Figure 5a through 5f reflects immunoassays on serum or plasma from different patients.

The data show that the measured concentration of troponin I in all of the serum and plasma samples tested was increased by the addition of binding inhibitors. Importantly, the time profile of the measured concentration of troponin I was in some cases biphasic (Figures 5a-c and 5e). The fast phase was complete within the first assay time of 0.5 h. The slow phase continued to rise for 6-24 hours depending on the sample. A slow phase was also observed for the purified ternary troponin complex (Example 13). The slow phase observed for the diluted serum and plasma samples, may, therefore, be associated with the opening up or partial unraveling of a ternary complex by the inhibitors and



antibodies. The fast phase indicates a bound complex of troponin I that is more easily opened up or partially unraveled than the ternary complex, since the fast phase is absent for the purified ternary complex (Example 13). Thus, the fast phase could be associated with the opening up or partial unraveling of binary complexes of troponin I.

Example 15

Immunoassays That are Sensitive To Free Troponin I, Troponin I Bound in a Ternary Complex, and Both Free and Bound Troponin I

Three sets of antibodies were evaluated for their ability to recognize free troponin I and troponin I bound in the ternary complex. Three antibody stock solutions described below as #1-3 were prepared in metal-free assay buffer either with or without binding inhibitors (30 mM EDTA and 0.15 mM Melittin) and the following antibodies:

1. 30 µg/ml 1A12 anti troponin I and 7.5 µg/ml biotinylated anti troponin I peptide 4 specific;
2. 30 µg/ml 1A12 anti troponin I, 30 µg/ml 9B1 anti troponin T monoclonal (Biospecific), 5 µg/ml each of biotinylated anti troponin I peptide 1, 2, 3 and 4 specific;
3. 30 µg/ml 9B1 anti troponin T and 5 µg/ml each of biotinylated anti troponin I peptide 1, 2, 3 and 4 specific.

Human cardiac troponin ternary complex (Bio-tech International, Inc.) was diluted to 1-15 ng/ml troponin I equivalents in metal free assay buffer and purified troponin I (Bio-tech International, Inc.) was diluted to 2, 4 and 8 ng/ml in the same buffer. The dilutions of the troponin complex and troponin I were assayed immediately using Protocol B, Example 2 with antibody solutions #1-3. Aliquots of the reaction mixtures formed by the addition of the

antibodies to the troponin complex and troponin I samples were taken at 0.5 h and 2.5 h after the antibodies were added and were further treated as described in Example 2, Protocol B. The results are shown in Table 7 and are expressed in terms of an assay slope with units of OD<sub>490</sub> per ng/ml total troponin I in the linear range of the assay. A higher slope indicates better binding of the antibodies to the troponin components. Antibody solution #2 was tested and found to be negative for cross reactivity with purified human cardiac troponin T (Scripps Labs) at 1-6 ng/ml using the assay protocol described herein (data not shown).

The data show that the immunoassay using the antibodies in solution #1 recognizes free troponin I but not troponin I in the ternary complex. The immunoassays using the antibodies in solution #2 recognizes free troponin I and troponin I in the ternary complex almost equally well. Thus, antibody solution #2 is superior to solution #1 for the assay of total troponin I when a fraction of the troponin I is present as the ternary complex. The immunoassay using the antibodies in solution #3 recognizes the ternary complex well but recognizes free troponin I poorly. This poor recognition of free troponin I causes the assay slope for antibody solution #3 to decrease over time in the presence of binding inhibitors. By using all three antibody solutions in immunoassays, one can estimate independently the concentrations of free troponin I (solution #1), total troponin I (solution #2) and bound troponin I (solution #3).

Table 7

Antibody solution #	Time after antibodies added (hours)	Assay Slope (OD <sub>490</sub> per ng/ml total troponin I)		
		Free Troponin I	Troponin ternary complex (without binding inhibitors)	Troponin ternary complex (with binding inhibitors)
#1	0.5	0.14	< 0.006	< 0.006
#1	2.5	0.14	0.003	0.021
#2	0.5	0.12	0.18	0.14
#2	2.5	0.22	0.17	0.12
#3	0.5	0.008	0.18	0.17
#3	2.5	0.008	0.17	0.08

Example 16

Estimation of Free Troponin I, Bound Troponin I and Total Troponin I in Plasma From a Patient with a Myocardial Infarction

The three antibody solutions described in Example 15 were used in immunoassays to measure the troponin I concentration in plasma from a patient with a confirmed myocardial infarction. The frozen plasma was treated and diluted into metal-free assay buffer as described in Example 14. The diluted plasma was assayed for troponin I immediately using Protocol B, Example 2 with antibody solutions #1-3 (Example 15). Aliquots were taken 0.5 h and 2.5 h after the antibodies were added to the samples to form the reaction mixtures and were further treated as described in Example 2, Protocol B. Either free troponin I at 1-4 ng/ml or the

troponin complex (Bio-tech International, Inc.) at 1-8 ng/ml troponin I in metal-free assay buffer was assayed and used to construct a standard curve of OD<sub>490</sub> as a function of total troponin I concentration for each antibody solution. The concentration of troponin I in the neat plasma sample, as shown in Table 8, measured by each antibody solution was determined using either the standard curve for free troponin I or for troponin I in the ternary complex in the absence of binding inhibitors as indicated in Table 8 and as described in Example 14. The ratio determined by dividing the assayed troponin I concentration with binding inhibitors by the concentration without inhibitors is also shown in Table 8.

The data show that the concentration of troponin I determined by immunoassay using antibody solution #1 is much more sensitive to the presence of binding inhibitors and thereby to the opening up or partial unraveling of the troponin complex than that determined using solution #2 or #3. The data of Example 15 suggest that antibody solution #1 measures mainly free troponin I, solution #2 measures both free troponin I and troponin I bound in the ternary complex and solution #3 measures mainly troponin I bound in the ternary complex. Thus, the conclusions from Example 15 taken together with the data in Table 8, indicate that substantial amounts of both free and bound troponin I are present in the diluted plasma sample. Among the three antibody solutions used in the immunoassays, solution #2 gives the largest assayed troponin I concentration, as expected, because the immunoassay using antibody solution #2 measures both free and bound troponin I. Thus, antibody solution #2 appears to provide the most comprehensive measure of troponin I in the plasma sample. Antibody solution #2 standardized with the purified troponin complex gave the most stable assay with

respect to inhibitor addition and assay incubation time. The decrease of troponin I concentration at 2.5 h measured with antibody solution #2 standardized with free Troponin I is due to an increase at 2.5 h of the slope of the standard curve.

5

Table 8

Antibody solution	Time after antibodies added, (hours)	Standard used to determine troponin I concentration	Troponin I concentration in plasma, (ng/ml)		Ratio
			Without binding inhibitors	With binding inhibitors	
#1	0.5	Troponin I	91	186	2.0
#1	2.5	Troponin I	83	251	3.0
#3	0.5	Ternary Troponin Complex	134	108	0.8
#3	2.5	Ternary Troponin Complex	87	65	0.74
#2	0.5	Troponin I	280	360	1.3
#2	2.5	Troponin I	172	220	1.3
#2	0.5	Ternary Troponin Complex	229	299	1.3
#2	2.5	Ternary Troponin Complex	223	286	1.3

10

15

Example 17

Immunoassay of Free Human Cardiac Troponin T and Troponin T  
in the Human Cardiac Ternary Complex

Two antibody stock solutions (#1 and 2) were prepared  
as described below in metal-free assay buffer either with or  
without binding inhibitors (30 mM EDTA and 0.15 mM Melittin)  
and with the following antibodies:

1. 30 µg/ml 1A12 anti troponin I, 30 µg/ml 9B1 anti troponin  
T and 7.5 µg/ml biotinylated anti troponin T peptide 3  
specific (Biospecific).
2. 30 µg/ml 9B1 anti troponin T and 7.5 µg/ml biotinylated  
anti troponin T peptide 3 specific.

Human cardiac troponin ternary complex (Bio-tech  
International, Inc.) was diluted to 1-20 ng/ml troponin T in  
metal free assay buffer and purified Human cardiac troponin T  
(Scripps Labs) was diluted to 1.5, 3.0 and 6.0 ng/ml in the  
same buffer. The dilutions of the troponin complex and  
troponin T were assayed immediately using Protocol B, Example  
2 with antibody solutions #1 and #2. Aliquots of the  
reaction mixtures formed by the addition of the antibodies to  
the troponin complex and troponin T samples were taken at  
0.5 h and 3.0 h after the antibodies were added and were  
further treated as described in Example 2, Protocol B. The  
results are shown in Table 9 and are expressed in terms of an  
assay slope with units of OD<sub>490</sub> per ng/ml total troponin T in  
the linear range of the assay. A higher slope indicates  
better binding of the antibodies to the troponin components.

The data show that the antibodies in solution #1  
recognize both free troponin T and troponin T bound in the  
ternary complex equally well. The antibodies in solution #2  
recognize free troponin T well but recognizes troponin T in  
the ternary complex poorly. Thus, antibody solution #1 is  
expected to provide the most comprehensive and accurate

measure of the total concentration of troponin T in human blood samples in which a substantial amount of the ternary complex is present.

5

Table 9

Antibody solution	Time after antibodies added to troponin sample (hours)	Assay slope (OD490 per ng/ml troponin T)			
		Free troponin T without binding inhibitors	Free troponin T with binding inhibitors	Troponin complex without binding inhibitors	Troponin complex with binding inhibitor
#1	0.5	0.069	0.078	0.061	0.070
#2	0.5	0.078	0.085	0.013	0.012
#2	3.0	>0.08	>0.08	0.018	0.023

10

Example 18

15

Use of Troponin C to Prevent Non-specific Binding of Troponin I to Filter Membranes

20

Oxidized cardiac troponin I (air oxidized, Example 4, University of Birmingham) at 100 ng/ml final concentration in human serum (Hybritech, Inc., San Diego) was incubated either with or without 100 ug/ml human cardiac troponin C (Bio-tech International, Inc.) at room temperature for 30 minutes. Two filter membranes, a CytoSep filter (Ahlstrom Filtration, Mount Holly Springs, PA) and a glass fiber filter (GB-100R, Micro Filtration Systems, Dublin, CA) were cut into

rectangles measuring 1.5 cm by 3.0 cm and were secured to a transparency film (catalog #pp2500, 3M, Austin, TX) by a piece of tape across the filters. The troponin I solutions with and without troponin C (300  $\mu$ l) were applied slowly to the top of the filters at one end and the solution migrated through the filter to the far end by wicking action. About 15  $\mu$ l of solution was collected from the far end with a plastic pipet tip. The collected solutions were diluted by factors of 20, 40 and 80 in assay buffer and assayed using Protocol A, Example 2 with TRI-7 F81 anti troponin I conjugate and biotinylated anti troponin I peptide 3 specific antibodies. Aliquots of the troponin I solutions with and without troponin C that had not been passed through the filters were also assayed and used to construct a standard curve from which the concentration of troponin I in the solution that had passed through the membrane was determined. The calculated concentration was divided by 100 ng/ml to obtain the fraction of recovered troponin I shown in Table 10. The experimental errors given in Table 10 represent one standard deviation.

The data show that the presence of troponin C helps to lower the non-specific binding of troponin I to the filter membranes.

Table 10

Filter	Fraction of troponin I recovered	
	Without troponin C	With troponin C
CytoSep	0.03 $\pm$ 0.03	0.15 $\pm$ 0.04
Glass Fiber	0.00 $\pm$ 0.03	0.09 $\pm$ 0.04



Example 19

Use of Proteins of High Isoelectric Point to Prevent Non-specific Binding of Troponin I to Filter Membranes

5 A blood filter (CytoSep 1.5 cm x 3.0 cm) was soaked  
for 16 hours at room temperature in solutions of deionized  
water containing 1 mg/ml of the proteins listed in Table 11.  
The filters were rinsed once with deionized water and dried  
for 2 hours at 35 C. Oxidized human cardiac troponin I (Bio-  
tech International, Inc.) at 100 ng/ml in human serum  
10 (Hybritech, Inc., San Diego, CA) also containing 2 mM added  
calcium chloride was passed through the filters as described  
in Example 18. The amount of troponin I recovered from the  
filters was determined by assay (Protocol B, Example 2) using  
TRI-7 F81 anti-troponin I and biotinylated anti troponin I  
15 peptide 4 specific antibodies with added binding inhibitors  
(Example 11). The data in Table 11 are expressed as the  
fraction of troponin I recovered, which was determined as  
described in Example 18.

20 The results show that Melittin and protamine  
sulfate substantially reduces the non-specific binding of  
troponin I to the blood filter, whereas casein and non-fat  
dried milk had little effect at the concentrations tested.

Table 11

Protein	Fraction of troponin I recovered
No addition	0.16
Protamine Sulfate	0.77
Casein	0.16
Melittin	0.72
Non-fat dried milk	0.08

Example 20

Immunoassay of Ternary Troponin Complex using TRI-7 F81 anti Troponin I and Biotinylated anti Troponin I Peptide 4 Antibodies

The purified ternary troponin complex (Bio-tech International, Inc.) was assayed for troponin I as described in Example 13, except that the title antibody pair was used in the immunoassay and the aliquot of the reaction mixture of the antibodies with the troponin sample was taken three hours after the antibodies were added to the troponin. The fractional assay response was 0.16 in the absence of binding inhibitors and 0.49 in the presence of binding inhibitors.

The data show that the title antibody pair recognizes troponin I in the ternary complex poorly. In example 10, it was shown that the presence of troponin C without Troponin T had little effect on the recognition of the title antibody pair for troponin I. Thus, the title antibody pair can bind to troponin I present in the binary complex with troponin C better than it can bind to Troponin I present in the ternary complex.

Example 21

Immunoassay of Troponin I in Plasma From a Patient With Confirmed Myocardial Infarction Using TRI-7 F81 Anti Troponin

I Conjugate and Biotinylated Anti Troponin I Peptide 1  
Specific Antibodies

Frozen plasma from a patient with a confirmed myocardial infarction was thawed and diluted in human serum (Hybritech Inc., San Diego, CA) also containing 0.5 M added sodium chloride and assayed for troponin I with the title antibody pair using Protocol A, Example 2. Oxidized purified human cardiac troponin I (University of Birmingham) was assayed and used to construct a standard curve from which the troponin I in the plasma was determined. The neat plasma sample was refrozen in a -70 C freezer after being on ice for several hours. The frozen plasma was rethawed at room temperature and was assayed using the same protocol as described above except the plasma and troponin I standards were diluted into assay buffer. The standards exhibited almost identical assay slopes when diluted in serum (first assay) or assay buffer (second assay). The neat plasma sample was further incubated at 4 C for the times indicated in Table 12 and reassayed in assay buffer.

The data show a substantial increase in the assayed troponin I concentration after a freeze/thaw cycle and after incubation at 4 C. This assay instability may be associated with the opening up or partial unraveling of the ternary troponin complex by the freeze/thaw cycle.

Table 12

Time of assay	Assayed concentration of troponin I
After first thaw of plasma	284 ng/ml
2 hours after second thaw of plasma	> 800 ng/ml
19 hours after second thaw of plasma	1760 ng/ml
90 hours after second thaw of plasma	2300 ng/ml

Example 22

Expression, Screening and Selection of Recombinant Antibodies

(Binding Fragments or Fab Fragments)

Immunization of mice

Mice were immunized by the following method. Each of ten mice were immunized intraperitoneally using 50  $\mu$ g protein antigen (troponin I and troponin ternary complex) emulsified in Freund's complete adjuvant on day 0, and day 28. Test bleeds of mice were obtained through puncture of the retro-orbital sinus. When the titers were determined to be high for biotinylated antigen, the mice were boosted with 50  $\mu$ g of protein on day 70, 71, and 72, with subsequent sacrifice and splenectomy on day 77. If titers of antibody were not satisfactory, mice were boosted with 50  $\mu$ g antigen on day 56 and a test bleed was obtained on day 63. If satisfactory titers were obtained the animals were boosted with 50  $\mu$ g of antigen on day 98, 99, and 100 and the spleens extracted on day 105.

Antibody phage display libraries

Mice having high titers of antibodies to the desired protein antigen (troponin I or troponin ternary complex) were sacrificed, and total RNA was purified from the spleen cells (Anal. Biochem. 162:156-159 (1987)). The total RNA (50  $\mu$ g) from the spleen cells was used directly as template for Superscript™ II reverse transcriptase (Gibco BRL, Gaithersburg, MD) with oligo (dT)<sub>12</sub> as the primer to make cDNA for PCR. A total of 32 PCR reactions was used to amplify the heavy chain variable regions using Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN), 3  $\mu$ L cDNA per reaction, 32 different 5' oligonucleotides and a single 3' oligonucleotide. The kappa chain variable regions were amplified similarly using 29 different 5' oligonucleotides and a single 3' oligonucleotide. The oligonucleotides were previously designed to ensure that nearly all antibody variable regions present in the spleen would be amplified with no more than 2 changes in the actual amino acid sequence caused by mismatches of the oligonucleotide with the cDNA based on a compilation of mouse antibody sequences. (Sequences of proteins of immunological interest. Vol. 2., 5th edition, 1991, Cambridge, MA.) An aliquot of each double stranded PCR product was amplified a second time using only the 3' oligonucleotide to produce single stranded DNA (ss-DNA). The ss-DNA products from all of the kappa chain amplification reactions were pooled, and the ss-DNA products from all of the heavy chain reactions were pooled separately. The ss-DNA from each pooled sample was purified using high performance liquid chromatography (HPLC) and a Genpak Fax HPLC column (Waters Inc, Milford, MA). The purified ss-DNA was used to perform mutagenesis of an M13 uracil template following standard oligonucleotide directed mutagenesis

techniques (J. Immunol. 149:3903-3913, 1992). The M13 template contains DNA sequences complementary to the 5' and 3' ends of the ss-DNA for both the heavy chain and the kappa chain variable regions. As a result, when the heavy chain and kappa chain variable regions were annealed to the M13 vector, a mixture of different antibody sequences was created. The resulting antibodies were expressed as Fab antibody fragments comprising the entire kappa chain and the variable region of the heavy chain linked to the first constant region of the heavy chain. Electrocompetent cells (DH12S Gibco BRL., Gaithersburg, MD) were transformed by electroporation with the resultant M13 DNA. A typical electroporation of 500 ng M13 vector into 40  $\mu$ L DH12S cells yielded  $10^7$  to  $10^8$  different antibody clones. The clones were further amplified to produce an antibody phage display library. The M13 vector was designed so that the heavy chain was expressed as a fusion protein with the major coat protein of M13, the gene VIII protein. There are approximately 2700 copies of the gene VIII protein on every phage particle. After the heavy chain/gene VIII fusion protein was secreted to the periplasm of *E. coli*, the secreted kappa chain binds to the heavy chain to form a functional antibody Fab fragment. This leads to the production of phage having functional antibodies on the surface of the phage with the DNA encoding the antibody packaged inside the phage.

#### Screening of antibody phage display libraries

The antibody phage display library obtained from electroporating the M13 mutagenesis DNA into *E. coli* consists of many different antibodies displayed on phage particles (antibody phage). The number of high affinity antibodies to the protein antigen is low. The following screening procedure was developed to isolate antibodies specific to

troponin I and the troponin ternary complex. Antibody phage was incubated with biotinylated, oxidized troponin I ( $10^{-9}$ M, 10 biotins/protein) or biotinylated troponin ternary complex ( $10^{-9}$ M, 11 biotins/protein) in solution to equilibrium. The antibody phage binding to biotinylated protein antigen was isolated by incubation with magnetic latex coated with avidin. The nonspecific antibody phage were washed away, and antibody phage binding to latex was eluted and amplified by growth in XL1 blue *E. coli* cells (Stratagene, La Jolla, CA). The amplified antibody phage were then subjected to a second round of selection. Since the incubation of biotinylated protein antigen and antibody phage was performed in solution, the concentration of biotinylated protein antigen was adjusted to select only for high affinity antibodies. The process described above was repeated until the antibody phage consisted of a high percentage of phage encoding troponin antibodies. The antibody sequences were then ready for subcloning.

#### Antibody subcloning

The entire antibody DNA sequence was amplified using a 5' oligonucleotide binding to the signal sequence of the kappa chain, which is on the 5' side of the heavy chain, and a 3' oligonucleotide binding to the end of the heavy chain constant region sequence. After amplification, the DNA was purified using agarose gel electrophoresis, then annealed and ligated to a pBR322 expression vector. Transformation of DH10B cells by electroporation was accomplished using this DNA, and the cells were grown on tetracycline plates to select clones containing the inserted DNA. Colonies were transferred into 3 mL 2YT media with 10  $\mu$ g/mL tetracycline, and cultures were grown overnight at 37°C. Overnight cultures of cells were diluted 1:100 into 50mL 2YT media with 1%

glycerol and 10mg/mL tetracycline in 250 mL baffled shake  
flasks to obtain sufficient antibody for testing. Cultures  
were grown at 37°C until the absorbance of the culture at 600  
nm was between 2 and 4. At that point, cultures were induced  
and grown overnight at 23°C. The cells were disrupted in a  
high pressure homogenizer, and the antibody was purified.

Antibody characterization by epitope mapping

The antibodies produced were characterized with  
regard to their ability to perform in a double antibody,  
solid-phase, noncompetitive enzyme immunoassay (sandwich  
assay). Antibody Fab fragments (Fab) were selected to bind  
distinctly different epitopes on the protein antigen so that  
the binding of one Fab did not sterically hinder the binding  
of the second Fab. The protein antigen was labeled with  
biotin by using biotin-XX-NHS ester (Molecular Probes,  
Portland, OR). The biotinylation of the protein antigen was  
sufficiently low in molar ratio of biotin to protein (on  
average, 4 biotin/protein) so that the probability of an  
epitope being substantially affected with regard to the  
native structure of the antibody binding site was minimal.  
The first Fab fragment was incubated in microtiter plate  
wells which contained adsorbed, affinity-purified goat-anti-  
mouse Fab. The wells were washed and a blocking solution  
containing a non-specific mouse Fab at high concentrations  
was placed in each well to saturate remaining anti-Fab sites.  
In separate microtiter plate wells, the second Fab fragment  
was incubated to equilibrium with the biotinylated protein  
antigen. The second Fab was present in substantial molar  
excess over the biotinylated protein antigen. The mixture  
containing the second Fab and the biotinylated protein was  
added to the microtiter plate wells that were coated with the  
first Fab and incubated. A conjugate of streptavidin and



alkaline phosphatase was added to the mixture to bind to the biotinylated protein to detect the presence of the protein antigen. The microtiter wells were washed and the presence of bound alkaline phosphatase was detected by adding phenolphthalein monophosphate, and the rate of product formation at 560 nm was determined using a spectrophotometric microtiter plate reader. The excess non-specific mouse Fab in the mixture prevented the second Fab from binding to anti-Fab adsorbed to the wells of the microtiter plate. Controls were performed using the same Fab as the first and second Fab to show that when the epitope bound by the second Fab is the same as the first, very little binding of the alkaline phosphatase to the microtiter plate well was observed. If the two different Fab fragments being tested can bind to different epitopes on the same protein, then the amount of alkaline phosphatase activity bound to the well was substantially greater than that of the control. The advantage of this assay procedure is that the antibodies were unlabeled so that many antibodies can be rapidly assayed to determine if they bind distinctly different epitopes. The number of distinct epitopes was determined and antibodies were grouped according to their epitope specificity.

#### Example 23

#### Characterization and Selection of Complementary Antibody Pairs For Recognition of Oxidized and Reduced Free troponin I and Troponin I bound in the ternary complex

Antibodies directed to troponin I were tested in a sandwich immunoassay in pairs consisting of an antibody conjugate antibody and a complementary biotinylated antibody (example 1). The antibodies tested were anti troponin I recombinant antibodies that were originally raised to free

troponin I but were selected to bind both free troponin I and troponin I in the ternary complex (example 22). Goat anti troponin I peptide 3 specific polyclonal antibody was also tested.

5                   Purified human cardiac ternary troponin complex (Bio-Tech International) was diluted into assay buffer and assayed (protocol A, Example 2) with the complementary antibody pairs shown in Table 13. The results are expressed as an assay slope with units of OD<sub>490</sub> per ng/ml total troponin  
10 I in the linear range of the assay. A higher slope indicates better binding of the antibodies to the troponin I.

                  Troponin I was prepared by dissociating the components of the ternary troponin complex under oxidizing (hereafter called dissociated/oxidized sample) and reducing  
15 (hereafter called dissociated/reduced sample) conditions. Purified human cardiac ternary troponin complex was incubated at 4 ug/ml for 4 hours at room temperature in a dissociation buffer consisting of 50 mM 3-(N-morpholino) propane sulfonic acid, 150 mM sodium chloride, 6 M urea, 10 mM EDTA and 0.05  
20 mM Melittin, pH 7.0, to form the dissociated/oxidized sample. The dissociated/oxidized sample was diluted to 1-30 ng/ml (total protein concentration) in assay buffer and assayed (protocol A, Example 2) with the complementary antibody pairs shown in Table 13. The same procedure was used to form and  
25 assay the dissociated/reduced sample except that the dissociation and assay buffers contained also 1.5 mM DTT to reduce the intramolecular disulfide of troponin I.

                  To show that the dissociation procedure did not harm free troponin I with respect to assays of free troponin  
30 I, purified oxidized troponin I (Bio-Tech International) was treated using the procedure to form the dissociated/oxidized sample and was then assayed with ten different complementary

antibody pairs (pairs 19-28 in Table 13). The assay results (not shown) were compared with those obtained on purified oxidized troponin I that was not treated with the dissociation procedure. No significant difference between the assay results for the treated and the untreated purified troponin I was observed for any of the ten tested antibody pairs.

The antibody pair (#29, Table 13) consisting of biotinylated 9B1 anti troponin T monoclonal and Goat anti troponin I peptide 3 specific polyclonal conjugate antibodies does not recognize troponin I that is dissociated from the ternary complex (Example 15), which is consistent with the zero assay slope obtained for this antibody pair with the dissociated/oxidized and dissociated/reduced samples.

The data (Table 13) show that antibodies directed to troponin I can be generated and selected to form an immunoassay that gives essentially the same assay response (slope) for troponin I in the ternary complex, the dissociated/oxidized and the dissociated/reduced samples (for example, antibody pair #17). Thus, an antibody pair such as #17 could be used to measure the total concentration of troponin I in a sample containing all of the forms of troponin I that were tested. The procedure used to generate and select the antibodies produces antibodies that are good candidates for use in an assay that measures oxidized and reduced free troponin I and troponin I in the ternary complex in the blood of patients who have suffered a myocardial infarction.

Pair #	Table 13 Conjugate Antibody	Biotinylated Antibody	Assay Slope (OD <sub>490</sub> per ng/ml troponin I)		
			Ternary Complex	Dissociated (oxidized)	Dissociated (reduced)
1	Goat anti troponin I Peptide 3 Specific	Recombinant anti troponin I #4	0.78	0.72	1.23
2	Goat anti troponin I Peptide 3 Specific	Recombinant anti troponin I #16	0.77	0.55	0.53
3	Goat anti troponin I Peptide 3 Specific	Recombinant anti troponin I #19/33	1.07	0.77	0.77
4	Goat anti troponin I Peptide 3 Specific	Recombinant anti troponin I #28/36	0.58	0.67	1.03
5	Goat anti troponin I Peptide 3 Specific	Recombinant anti troponin I #49	0.73	0.51	0.52
6	Goat anti troponin I Peptide 3 Specific	Recombinant anti troponin I #50	0.70	0.61	0.98
7	Goat anti troponin I Peptide 3 Specific	Recombinant anti troponin I #51	0.89	0.66	0.69
8	Goat anti troponin I Peptide 3 Specific	Recombinant anti troponin I #53	0.55	0.86	0.89
9	Goat anti troponin I Peptide 3 Specific	Recombinant anti troponin I #26	0.21	0.12	0.18
10	Goat anti troponin I Peptide 3 Specific	Recombinant anti troponin I #27	0.54	0.38	0.46
11	Goat anti troponin I Peptide 3 Specific	Recombinant anti troponin I #29	0.78	0.58	0.60

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12	Goat anti troponin I Peptide 3 Specific	Recombinant anti troponin I #31	0.60	0.52	0.64
13	Goat anti troponin I Peptide 3 Specific	Recombinant anti troponin I #32	0.78	0.64	0.58
14	Goat anti troponin I Peptide 3 Specific	Recombinant anti troponin I #54	0.23	0.54	0.45
15	Goat anti troponin I Peptide 3 Specific	Recombinant anti troponin I #55	0.23	0.52	0.47
16	Goat anti troponin I Peptide 3 Specific	Recombinant anti troponin I #56	0.69	0.99	1.05
17	Goat anti troponin I Peptide 3 Specific	Recombinant anti troponin I #57	1.02	1.08	1.08
18	Goat anti troponin I Peptide 3 Specific	Recombinant anti troponin I #58	0.39	0.90	0.95
19	Recombinant anti troponin I #49	Recombinant anti troponin I #4	0.48	0.52	1.06
20	Recombinant anti troponin I #49	Recombinant anti troponin I #28/36	0.21	0.41	0.96
21	Recombinant anti troponin I #49	Goat anti Troponin I Peptide 3 Specific	1.18	1.12	1.25
22	Recombinant anti troponin I #51	Recombinant anti troponin I #4	2.42	3.31	7.63
23	Recombinant anti troponin I #51	Recombinant anti troponin I #28/36	1.20	2.52	5.69
24	Recombinant anti troponin I #50	Recombinant anti troponin I #16	1.33	0.53	1.20

25	Recombinant anti troponin I #50	Recombinant anti troponin I #19/33	1.93	0.81	1.95
26	Recombinant anti troponin I #50	Goat anti Troponin I Peptide 3 Specific	1.92	1.52	2.12
27	Recombinant anti troponin I #53	Recombinant anti troponin I #16	1.58	3.33	3.49
28	Recombinant anti troponin I #53	Recombinant anti troponin I #33/79	1.60	3.75	4.28
29	Goat anti Troponin I Peptide 3 Specific	9B1 anti troponin T monoclonal	0.47	0.00	0.00

Example 24

Effect of Protamine and Melittin on the Non-Specific  
Absorption of Troponin to Latex Particles

Avidin-HS coated magnetic latex (Example 1) was  
5 washed and resuspended to 1% solids in three different  
diluent: serum (Hybritech Inc., San Diego, CA), serum  
containing 0.2 mg/ml protamine chloride and serum containing  
0.1 mM melittin. A 125  $\mu$ l volume of each resuspended latex  
solution was mixed with a 125  $\mu$ l volume of serum containing  
10 either 6 ng/ml of oxidized purified troponin I (Bio-Tech  
International, Inc) or 18 ng/ml of purified ternary troponin  
complex to form a solution of troponin and latex. Also, a 125  
 $\mu$ l volume of serum, serum containing 0.2 mg/ml protamine  
chloride or serum containing 0.1 mM Melittin was mixed with a  
15 125  $\mu$ l volume of serum containing either 6 ng/ml of oxidized  
purified troponin I or 18 ng/ml of purified ternary complex  
to form a solution of troponin without latex. The solutions  
were incubated for 30 minutes at room temperature. The latex  
was pelleted and the supernatants collected. The supernatants  
20 from the solutions of troponin and latex and the solutions of  
troponin without latex were assayed for troponin (Protocol A,  
Example 2, except serum was used in place of assay buffer)  
using antibody pair #17 (Table 13) to determine the  
concentration of troponin. For each diluent, the fraction of  
25 troponin recovered in the supernatants of the solutions of  
troponin and latex (Table 14) was determined by dividing the  
measured concentration of troponin in the supernatant from  
the solution of troponin and latex by the measured  
concentration of troponin in the solution of troponin without  
30 latex.

The data show that both melittin and protamine  
chloride increase the recovery of troponin, indicating that

melittin and protamine chloride reduce the non-specific absorption of troponin to the latex.

Table 14

Troponin Form	Fraction of Troponin Recovered in the Supernatant		
	No Addition	+ Protamine Chloride	+ Melittin
Free Oxidized Troponin I	0.49	0.73	0.80
Troponin Complex	0.58	0.87	0.77

Example 25

Effect of Protamine and Melittin on the Assay Response of a Sandwich Immunoassay For Troponin That Utilizes Latex Particles

Avidin-HS magnetic latex (Example 1) was washed and resuspended to 1% solids in two different diluents: assay buffer and assay buffer containing 0.1 mM melittin. In the wells of a microtiter plate, 25  $\mu$ l of each resuspended latex solution was mixed with 25  $\mu$ l of assay buffer containing purified oxidized troponin I (Bio-Tech International) at various concentrations between 0 and 16 ng/ml to form a solution of troponin I and latex. The solutions were incubated at room temperature for 30 minutes. To each well was added 50  $\mu$ l of a solution consisting of the two antibodies of pair #17 (Table 13), each at 2.5 ug/ml, to form a reaction mixture. The reaction mixture was incubated for 15 minutes and the latex was then pelleted, washed and further treated as described in Protocol A (Example 2). The results



are expressed in Table 15 as an assay slope with units of OD<sub>490</sub> per ng/ml troponin I.

The data show that the addition of melittin to the assay increases the assay response by about a factor of two, thus increasing assay sensitivity to troponin I. In data not shown, incorporation of protamine (at 0.03-0.1 mg/ml) and melittin (at 0.017-0.05 mM) into an immunoassay for troponin I that utilized latex particles increased the assay response by factors of 30% to 400%.

Table 15

Additive	Assay Slope
None	0.021
Melittin	0.040

We claim:

1. An immunoassay system for determining the presence or amount of a troponin form or a group of troponin forms in a whole blood, plasma or serum sample suspected of containing troponin from damaged heart muscle, said system comprising:

a) formation of an antibody conjugate comprising an antibody coupled to a signal generating element, said antibody capable of specifically binding to cardiac specific regions of a form of troponin or a group of troponin forms;

b) formation of a reaction mixture comprising said whole blood, plasma or serum sample incubated with said antibody conjugate;

c) application of said reaction mixture to a surface to which is bound at least one capture antibody capable of specifically binding to cardiac specific regions of a form of troponin or a group of troponin forms in said antibody conjugate, said capture antibody binding said antibody conjugate, whereby the immobilized conjugate produces a detectable signal upon formation of sandwich complexes; and,

d) relation of detectable signal to the presence or amount of said troponin form or said group of troponin forms in said sample.

2. The immunoassay system of claim 1 wherein the conjugate antibody of step a) is a sensitive antibody which comprises the ability to specifically bind a single troponin form; and step d) comprises relation of detectable signal to the presence or amount of said single troponin form in said sample.

3. The immunoassay system of claim 1 wherein the capture antibody of step c) is a sensitive antibody which comprises the ability to specifically bind a single troponin form; and step d) comprises relation of detectable signal to the presence or amount of said single troponin form in said sample.

4. The immunoassay system of claim 1 wherein the conjugate antibody of step a) is an insensitive antibody and specifically binds at least two troponin forms; and step d) comprises relation of detectable signal to the presence or amount of a group of troponin forms in said sample.

5. The immunoassay system of claim 1 wherein the capture antibody of step c) is an insensitive antibody and specifically binds at least two troponin forms; and step d) comprises relation of detectable signal to the presence or amount of a group of troponin forms in said sample.

6. The immunoassay system of claim 1 for  
determining the presence or amount of troponin I in a whole  
blood, plasma or serum sample suspected of containing  
troponin complexes or components from damaged heart muscle,  
said system comprising:

a) formation of an antibody conjugate comprising an  
antibody coupled to a signal generating element,  
said antibody capable of binding to cardiac  
specific regions of troponin I complexes and to  
unbound troponin I;

b) formation of a reaction mixture comprising said  
whole blood, plasma or serum sample incubated with  
said antibody conjugate;

c) application of said reaction mixture to a solid  
phase to which is bound at least one capture  
antibody complementary to the antibody conjugate,  
said capture antibody binding said antibody  
conjugate, whereby the immobilized conjugate  
produces a detectable signal upon formation of  
sandwich complexes between troponin I complexes or  
unbound troponin I; and,

d) relation of detectable signal to the presence or  
amount of troponin I in said sample.

7. The immunoassay system of claim 1 for  
determining the presence or amount of troponin I in a whole  
blood, plasma or serum sample suspected of containing  
5 troponin complexes or components from damaged heart muscle,  
said system comprising:

10 a) formation of an antibody conjugate comprising an  
antibody coupled to a signal generating element,  
said antibody capable of binding to the oxidized  
form of troponin I or to the reduced form of  
troponin I;

15 b) formation of a reaction mixture comprising said  
whole blood, plasma or serum sample incubated with  
said antibody conjugate;

20 c) application of said reaction mixture to a solid  
phase to which is bound at least one capture  
antibody complementary to the antibody conjugate,  
said capture antibody binding said antibody  
conjugate, whereby the immobilized conjugate  
produces a detectable signal upon formation of  
sandwich complexes between troponin I complexes or  
25 unbound troponin I; and,

d) relation of detectable signal to the presence or  
amount of troponin I in said sample.

30 8. The immunoassay of claim 7 wherein the antibody  
of step a) is capable of binding to the oxidized form of  
troponin I and to the reduced form of troponin I.

9. The immunoassay of claim 7 wherein the antibody of step a) is capable of binding to the oxidized form of troponin I or to the reduced form of troponin I where said troponin I is unbound or in a complex.

10. The immunoassay system of claim 1 for  
determining the presence or amount of troponin T in a whole  
blood, plasma or serum sample suspected of containing  
5 troponin complexes or components from damaged heart muscle,  
said system comprising:

10 a) formation of an antibody conjugate comprising an  
antibody coupled to a signal generating element,  
said antibody capable of binding to cardiac  
specific regions of troponin T complexes and to  
unbound troponin T;

15 b) formation of a reaction mixture comprising said  
whole blood, plasma or serum sample incubated with  
said antibody conjugate;

20 c) application of said reaction mixture to a solid  
phase to which is bound at least one capture  
antibody complementary to the antibody conjugate,  
said capture antibody binding said antibody  
conjugate, whereby the immobilized conjugate  
produces a detectable signal upon formation of  
sandwich complexes between troponin T complexes or  
25 unbound troponin T; and,

d) relation of detectable signal to the presence or  
amount of troponin T in said sample.

11. The immunoassay system of claim 1 for  
determining the presence or amount of bound and unbound  
troponin T in a whole blood, plasma or serum sample suspected  
of containing troponin complexes or components from damaged  
heart muscle, said system comprising:

a) formation of an antibody conjugate composition  
comprising at least two antibodies each coupled to  
a signal generating element, at least one of said  
antibodies capable of binding to the cardiac  
specific regions of troponin T complexes and at  
least one of said antibodies capable of binding to  
unbound troponin T;

b) formation of a reaction mixture comprising said  
whole blood, plasma or serum sample incubated with  
said antibody conjugate;

c) application of said reaction mixture to a solid  
phase to which is bound at least one capture  
antibody complementary to each antibody of the  
antibody conjugate, said capture antibody binding  
the bound troponin:antibody conjugate complex to  
which it is complementary, whereby the immobilized  
conjugate produces a detectable signal upon  
formation of sandwich complexes between troponin T  
complexes or unbound troponin T; and,

d) relation of detectable signal to the presence or  
amount of troponin T in said sample.



12. The immunoassay system of claim 1 for  
determining the presence or amount of bound and unbound  
troponin I in a whole blood, plasma or serum sample suspected  
of containing troponin complexes or components from damaged  
heart muscle, said system comprising:

a) formation of an antibody conjugate composition  
comprising at least two antibodies each coupled to  
a signal generating element, at least one of said  
antibodies capable of binding to the cardiac  
specific regions of troponin I complexes and at  
least one of said antibodies capable of binding to  
unbound troponin I;

b) formation of a reaction mixture comprising said  
whole blood, plasma or serum sample incubated with  
said antibody conjugate;

c) application of said reaction mixture to a solid  
phase to which is bound at least one capture  
antibody complementary to each antibody of the  
antibody conjugate, said capture antibody binding  
the bound troponin:antibody conjugate complex to  
which it is complementary, whereby the immobilized  
conjugate produces a detectable signal upon  
formation of sandwich complexes between troponin I  
complexes or unbound troponin I; and,

d) relation of detectable signal to the presence or  
amount of troponin I in said sample.

13. The immunoassay system of claim 1 for  
determining the presence or amount of troponin I in a whole  
blood, plasma or serum sample suspected of containing unbound  
troponin I, troponin I binary complexes, and ternary  
5 complexes, said system comprising:

10 a) formation of an antibody conjugate composition  
comprising at least two antibodies each coupled to  
a signal generating element, at least one of said  
antibodies capable of binding to the troponin T  
component of the ternary complexes and at least one  
of said antibodies capable of binding to unbound  
troponin I and to binary troponin I molecules,  
wherein said antibody conjugate binds to ternary  
15 complexes through the troponin T specific antibody  
and binds unbound troponin I and binary troponin I  
molecules through the troponin I specific antibody;

20 b) formation of a reaction mixture comprising said  
sample incubated with said antibody conjugate;

25 c) application of said reaction mixture to a solid  
phase to which is bound in one zone at least one  
capture antibody complementary to each antibody of  
the antibody conjugate, said capture antibody  
binding the bound troponin:antibody conjugate  
complex to which it is complementary, whereby the  
immobilized conjugate produces a detectable signal  
upon binding unbound troponin I or troponin  
30 complexes containing troponin I; and,

d) relation of detectable signal to the presence or amount of troponin I in said sample.

14. The immunoassay system of claim 1 for determining the presence or amount of troponin T in a whole blood, plasma or serum sample suspected of containing unbound troponin T, troponin T binary complexes, and ternary complexes, said system comprising:

a) formation of an antibody conjugate composition comprising at least two antibodies each coupled to a signal generating element, at least one of said antibodies capable of binding to the troponin I component of the ternary complexes and at least one of said antibodies capable of binding to unbound troponin T and to binary troponin T molecules, wherein said antibody conjugate binds to ternary complexes through the troponin I specific antibody and binds unbound troponin T and binary troponin T molecules through the troponin T specific antibody;

b) formation of a reaction mixture comprising said sample incubated with said antibody conjugate;

c) application of said reaction mixture to a solid phase to which is bound in one zone at least one capture antibody complementary to each antibody of the antibody conjugate, said capture antibody binding the bound troponin:antibody conjugate complex to which it is complementary, whereby the immobilized conjugate produces a detectable signal upon binding unbound troponin T or troponin complexes containing troponin T; and,

d) relation of detectable signal to the presence or amount of troponin T in said sample.

15. The immunoassay system of claim 1 for determining the presence or amount, in a distinct zone or zones, of troponin I in a whole blood, plasma or serum sample, said system comprising:

5

a) formation of an antibody conjugate comprising an antibody coupled to a signal generating element, said antibody capable of binding to troponin I when bound in troponin complexes and to unbound troponin I;

10

b) formation of a reaction mixture comprising said serum sample incubated with said antibody conjugate;

15

c) application of said reaction mixture to a solid phase having at least one discrete zone, said zone comprising at least one immobilized capture antibody capable of binding complexes of troponin I or unbound troponin I; whereby a detectable signal is generated upon the formation of a sandwich complex in said discrete zone; and,

20

d) relation of detectable signal to the presence or amount of troponin I in said zone.

25

16. The system of claim 15 wherein step c) comprises application of said reaction mixture to a solid phase having at least two discrete zones, said first zone comprising at least one immobilized capture antibody capable of binding complexes of troponin I, and said second zone comprising at least one immobilized capture antibody capable of binding unbound troponin I, whereby a detectable signal is generated upon the formation of a sandwich complex in any one of said two zones; and, step d) comprises relation of detectable signal to the presence or amount of troponin I in said zones, whereby the total amount of troponin in said zones corresponds to the total amount of troponin in said sample.

17. The system of claim 15 wherein step c) comprises application of said reaction mixture to a solid phase having at least three discrete zones, said first zone comprising at least one immobilized capture antibody capable of binding ternary complex, said second zone comprising at least one immobilized capture antibody capable of binding binary complexes of troponin I, and said third zone comprising at least one immobilized capture antibody capable of binding unbound troponin I, whereby a detectable signal is generated upon the formation of a sandwich complex in any one of said three zones; and, step d) comprises relation of detectable signal to the presence or amount of troponin I in said zones, whereby the total amount of troponin in said zones corresponds to the total amount of troponin in said sample.

18. The system of claim 15 wherein said capture antibodies are specific to the interfaces of the binding domains of troponin I/T and I/C.

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19. The immunoassay system of claim 1 for determining the presence or amount, in a distinct zone or zones, of troponin T in a whole blood, plasma or serum sample, said system comprising:

5

a) formation of an antibody conjugate comprising an antibody coupled to a signal generating element, said antibody capable of binding to troponin T when bound in troponin complexes and to unbound troponin T;

10

b) formation of a reaction mixture comprising said sample incubated with said antibody conjugate;

15

c) application of said reaction mixture to a solid phase having at least one discrete zone, said zone comprising at least one immobilized capture antibody capable of binding complexes of troponin T or unbound troponin T; wherein a detectable signal is generated upon the formation of a sandwich complex in said discrete zone; and,

20

d) relation of detectable signal to the presence or amount of troponin T in said zone.

25



20. The system of claim 19 wherein step c) comprises application of said reaction mixture to a solid phase having at least two discrete zones, said first zone comprising at least one immobilized capture antibody capable of binding complexes of troponin T, and said second zone comprising at least one immobilized capture antibody capable of binding unbound troponin T, whereby a detectable signal is generated upon the formation of a sandwich complex in any one of said two zones; and, step d) comprises relation of detectable signal to the presence or amount of troponin I in said zones, whereby the total amount of troponin in said zones corresponds to the total amount of troponin in said sample.

21. The system of claim 20 wherein said capture antibodies are specific to the interfaces of the binding domains of troponin T/I and T/C.

22. The system of claim 19 wherein step c) comprises application of said reaction mixture to a solid phase having at least three discrete zones, said first zone comprising at least one immobilized capture antibody capable of binding ternary complex, said second zone comprising at least one immobilized capture antibody capable of binding binary complexes of troponin T, and said third zone comprising at least one immobilized capture antibody capable of binding unbound troponin T, whereby a detectable signal is generated upon the formation of a sandwich complex in any one of said three zones; and, step d) comprises relation of detectable signal to the presence or amount of troponin I in said zones, whereby the total amount of troponin in said zones corresponds to the total amount of troponin in said sample.

23. The system of claim 1 wherein a troponin inhibitor is added to said sample prior to formation of said reaction mixture so that said assay system predominantly measures unbound troponin.

24. The system of claim 23 wherein said inhibitor is selected from the group consisting of metal chelates, mastoparan and melittin.

25. The system of claim 1 wherein troponin C is added to said sample prior to the formation of said reaction mixture so that all or substantially all of troponin I or T being measured in said sample will be bound by the troponin C during the course of the assay.

26. The system of claim 1 wherein troponin C and T are added to said sample prior to the formation of said reaction mixture so that all or substantially all of troponin I in the form of the ternary troponin complex is bound during the course of the assay.

5

27. The system of claim 1 wherein troponin C and I are added to said sample prior to the formation of said reaction mixture so that all or substantially all of troponin T in the form of the ternary troponin complex is bound during the course of the assay.

10

28. The system of claim 1 wherein said antibody conjugate comprises a cocktail of antibodies each coupled to a signal generating element and where a cocktail of capture antibodies are bound to said surface.

15

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29. The immunoassay system of claim 1 for determining the presence or amount of troponin components in a whole blood, plasma or serum sample suspected of containing troponin from damaged heart muscle, said system comprising:

5

10

a) formation of an antibody conjugate comprising an antibody coupled to a signal generating element, said antibody being one that yields an assay response that is insensitive with regard to the form of troponin I, said antibody being capable of binding to cardiac specific regions of troponin I complexes and to unbound troponin I;

15

b) formation of a reaction mixture comprising said sample incubated with said antibody conjugate;

20

c) application of said reaction mixture to a solid phase to which is bound at least one capture antibody complementary to the antibody conjugate, said capture antibody binding said antibody conjugate, whereby the immobilized conjugate produces a detectable signal upon formation of sandwich complexes between troponin I complexes or unbound troponin I; and,

25

d) relation of detectable signal to the presence or amount of troponin I in said sample.

30. The immunoassay system of claim 29 wherein  
step a) comprises forming a conjugate with an antibody  
yielding an assay response that is insensitive with regard to  
the unbound oxidized and unbound reduced forms of troponin I  
and ternary complex; and, step d) comprises relation of  
detectable signal to the presence or amount of unbound  
oxidized and unbound reduced troponin I and ternary complex  
in said sample.

31. The immunoassay system of claim 30 wherein  
step a) comprises forming a conjugate with an antibody  
yielding an assay response that is insensitive with regard to  
the unbound oxidized and unbound reduced forms of troponin I;  
and, step d) comprises relation of detectable signal to the  
presence or amount of unbound oxidized and unbound reduced  
troponin I in said sample.

32. The immunoassay system of claim 30 wherein  
step a) comprises forming a conjugate with an antibody  
yielding an assay response that is insensitive with regard to  
the unbound oxidized form of troponin I and ternary complex;  
and, step d) comprises relation of detectable signal to the  
presence or amount of unbound oxidized troponin I and ternary  
complex in said sample.

33. The immunoassay system of claim 1 for determining the presence or amount of troponin components in a whole blood, plasma or serum sample suspected of containing troponin from damaged heart muscle, said system comprising:

5

a) formation of an antibody conjugate comprising an antibody coupled to a signal generating element, said antibody being one that yields an assay response that is insensitive with regard to the complexed or unbound forms of troponin T;

10

b) formation of a reaction mixture comprising said sample incubated with said antibody conjugate;

15

c) application of said reaction mixture to a solid phase to which is bound at least one capture antibody complementary to the antibody conjugate, said capture antibody binding said antibody conjugate, whereby the immobilized conjugate produces a detectable signal upon formation of sandwich complexes between troponin T complexes or unbound troponin T; and,

20

d) relation of detectable signal to the presence or amount of troponin T in said sample.

25

34. The immunoassay system of claim 33 wherein  
step a) comprises forming a conjugate with an antibody  
yielding an assay response that is insensitive with regard to  
the unbound form of troponin T and ternary complex; and, step  
5 d) comprises relation of detectable signal to the presence or  
amount of unbound troponin T and ternary complex in said  
sample.

35. The immunoassay system of claim 1 for determining the presence or amount of troponin components in a whole blood, plasma or serum sample suspected of containing troponin from damaged heart muscle, said system comprising:

5

10

a) formation of an antibody conjugate composition, said composition comprising a cocktail of sensitive antibodies, whereby said cocktail yields an assay response that is insensitive with regard to the forms of troponin I, said composition comprising antibodies capable of binding to cardiac specific regions of troponin I complexes and to unbound troponin I;

15

b) formation of a reaction mixture comprising said sample incubated with said antibody conjugate composition;

20

25

c) application of said reaction mixture to a solid phase to which is bound at least one capture antibody complementary to each antibody of the antibody conjugate, said capture antibody binding the bound troponin:antibody conjugate complex to which it is complementary, whereby the immobilized conjugate produces a detectable signal upon formation of sandwich complexes between troponin I complexes or unbound troponin I; and,

30

d) relation of detectable signal to the presence or amount of troponin I in said sample.



36. The immunoassay system of claim 35 wherein  
step a) comprises forming a conjugate with an antibody  
yielding an assay response that is insensitive with regard to  
the unbound oxidized and unbound reduced forms of troponin I  
and ternary complex; and, step d) comprises relation of  
detectable signal to the presence or amount of oxidized and  
reduced troponin I in said sample.

37. The immunoassay system of claim 36 wherein  
step a) comprises forming a conjugate with an antibody  
yielding an assay response that is insensitive with regard to  
the unbound oxidized and unbound reduced forms of troponin I;  
and, step d) comprises relation of detectable signal to the  
presence or amount of oxidized and reduced troponin I in said  
sample.

38. The immunoassay system of claim 36 wherein  
step a) comprises forming a conjugate with an antibody  
yielding an assay response that is insensitive with regard to  
the unbound oxidized form of troponin I and ternary complex;  
and, step d) comprises relation of detectable signal to the  
presence or amount of unbound oxidized troponin I and ternary  
complex in said sample.

39. The immunoassay system of claim 1 for determining the presence or amount of troponin components in a whole blood, plasma or serum sample suspected of containing troponin from damaged heart muscle, said system comprising:

5

a) formation of an antibody conjugate composition, said composition comprising a cocktail of sensitive antibodies, whereby said cocktail yields an assay response that is insensitive with regard to the unbound or complexed forms of troponin T;

10

b) formation of a reaction mixture comprising said sample incubated with said antibody conjugate composition;

15

c) application of said reaction mixture to a solid phase to which is bound at least one capture antibody complementary to each antibody of the antibody conjugate, said capture antibody binding the bound troponin:antibody conjugate complex to which it is complementary, whereby the immobilized conjugate produces a detectable signal upon formation of sandwich complexes between troponin T complexes or unbound troponin T; and,

20

25

d) relation of detectable signal to the presence or amount of troponin T in said sample.

40. The immunoassay system of claim 39 wherein  
step a) comprises forming a conjugate with an antibody  
yielding an assay response that is insensitive with regard to  
unbound form of troponin T and ternary complex; and, step d)  
5 comprises relation of detectable signal to the presence or  
amount of unbound troponin T and ternary complex in said  
sample.

41. The immunoassay system of claim 1 for independently determining the presence or amount of: 1) the cardiac ternary complex; 2) the cardiac troponin binary complex of I(oxidized)/T; 3) the cardiac troponin binary complex of I(reduced)/T; 4) the cardiac troponin binary complex of I(oxidized)/C; 5) the cardiac troponin binary complex of I(reduced)/C; 6) the cardiac troponin binary complex T/C; 7) unbound cardiac troponin I (oxidized); 8) unbound cardiac troponin I (reduced) and 9) unbound cardiac troponin T, in a whole blood, plasma or serum sample suspected of containing troponin from damaged heart muscle, said system capable of determining the presence of n forms of troponin, where n is a whole integer of from 1 to 9, said system comprising:

a) formation of an antibody conjugate composition comprising an antibody that binds troponin, wherein said antibody is coupled to a signal generating element;

b) formation of a reaction mixture comprising said sample incubated with said antibody conjugate;

c) application of said reaction mixture to a solid phase having n discrete zones, wherein the discrete zone comprises:

1) a zone comprising an immobilized capture antibody capable of binding said cardiac ternary complex;

2) a zone comprising an immobilized capture antibody capable of binding said cardiac troponin binary complex I (oxidized)/T;

3) a zone comprising an immobilized capture antibody capable of binding said cardiac troponin binary complex I(reduced)/T;

4) the cardiac troponin binary complex of I(oxidized)/C;

5) the cardiac troponin binary complex of I(reduced)/C; 6) a zone comprising an immobilized capture antibody capable of binding said cardiac troponin binary complex T/C; 7) a zone comprising an immobilized capture antibody capable of binding said unbound cardiac troponin I (oxidized);

8) a zone comprising an immobilized capture antibody capable of binding said unbound cardiac troponin I (reduced); or,

9) a zone comprising an immobilized capture antibody capable of binding said unbound cardiac troponin T, whereby a detectable signal is generated upon the formation of a sandwich complex in said discrete zone; and,

d) relation of detectable signal to the presence or amount of n forms of troponin from damaged heart muscle.

42. The immunoassay system of claim 41 wherein step a) further comprises that the antibody that binds troponin is an antibody that yields an assay response that is insensitive with regard to the form of troponin.

43. The immunoassay system of claim 42 wherein  
step c) further comprises that the immobilized capture  
antibody of said discrete zone is an antibody that yields an  
assay response which is sensitive with regard to the form of  
troponin.

5

44. The immunoassay system of claim 41 wherein  
step a) further comprises that the antibody that binds  
troponin is an antibody that yields an assay response that is  
sensitive with regard to the form of troponin.

10

45. The immunoassay system of claim 44 wherein  
step c) further comprises that the immobilized capture  
antibody of said discrete zone is an antibody that yields an  
assay response which is insensitive with regard to the form  
of troponin.

15

46. The immunoassay system of claim 1 for determining the presence or amount of troponin C in a whole blood, plasma or serum sample suspected of containing troponin from damaged heart muscle, said system comprising:

5

a) formation of an antibody conjugate comprising an antibody coupled to a signal generating element, said antibody capable of binding to cardiac specific regions of

10

troponin C;

b) formation of a reaction mixture comprising said sample incubated with said antibody conjugate;

15

c) application of said reaction mixture to a solid phase to which is bound at least one capture antibody complementary to the antibody conjugate, said capture antibody binding said antibody

20

conjugate, whereby the immobilized conjugate produces a detectable signal upon formation of sandwich complexes comprising troponin C; and,

d) relation of detectable signal to the presence or amount of troponin C in said sample.

47. A stabilized composition of troponin.

48. The stabilized composition of claim 47  
comprising a stabilized composition of troponin I, wherein  
5 the troponin I is oxidized.

49. The stabilized composition of claim 48 wherein  
the troponin I is unbound or the troponin I is in a complex.

10 50. The stabilized composition of claim 49  
comprising a stabilized composition of the ternary complex of  
troponin I, T and C.

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51. A method for improving the recovery of troponin I or T from a surface used in immunoassays, said method comprising: contacting with said surface at least one strongly basic peptide, protein, or polymer with a pI value greater than about 8.

5

52. The method of claim 51, further comprising a step of washing unbound peptide, protein or polymer from said surface.

10

53. The method of claim 51 wherein melittin is the strongly basic peptide used.

54. The method of claim 51 wherein protamine is the strongly basic protein used.

15

Novel Methods for the Assay of Troponin I and T and Complexes  
of Troponin I and T and Selection of Antibodies for Use in  
Immunoassays

5

ABSTRACT OF THE DISCLOSURE

Assay systems and specialized antibodies for the  
detection and quantitation of troponin I and troponin T in  
10 body fluids as an indicator of myocardial infarction. Since  
troponin I and T exist in various conformations in the blood,  
the ratios of the monomeric troponin I and T and the binary  
and ternary complexes, as well as which form of troponin  
present in the blood, may be related to the metabolic state  
15 of the heart. Disclosed is a system to determine the  
presence of a troponin form or a group of troponin forms in a  
sample of whole blood, serum or plasma.

Disclosed is a stabilized composition of troponin;  
the stabilized composition can comprise a stabilized  
20 composition of troponin I, wherein the troponin I is  
oxidized, the troponin I can be unbound or the troponin I can  
be in a complex.

Disclosed is a method for improving the recovery of  
troponin I or T from a surface used in immunoassays

25 Also disclosed are antibodies which recognize,  
unbound troponin forms, the forms of troponin in binary  
complexes, the ternary complex of troponin I, T and C, and  
the conformations of troponin I having intramolecularly  
oxidized and reduced cysteines.

30

Figure 1a

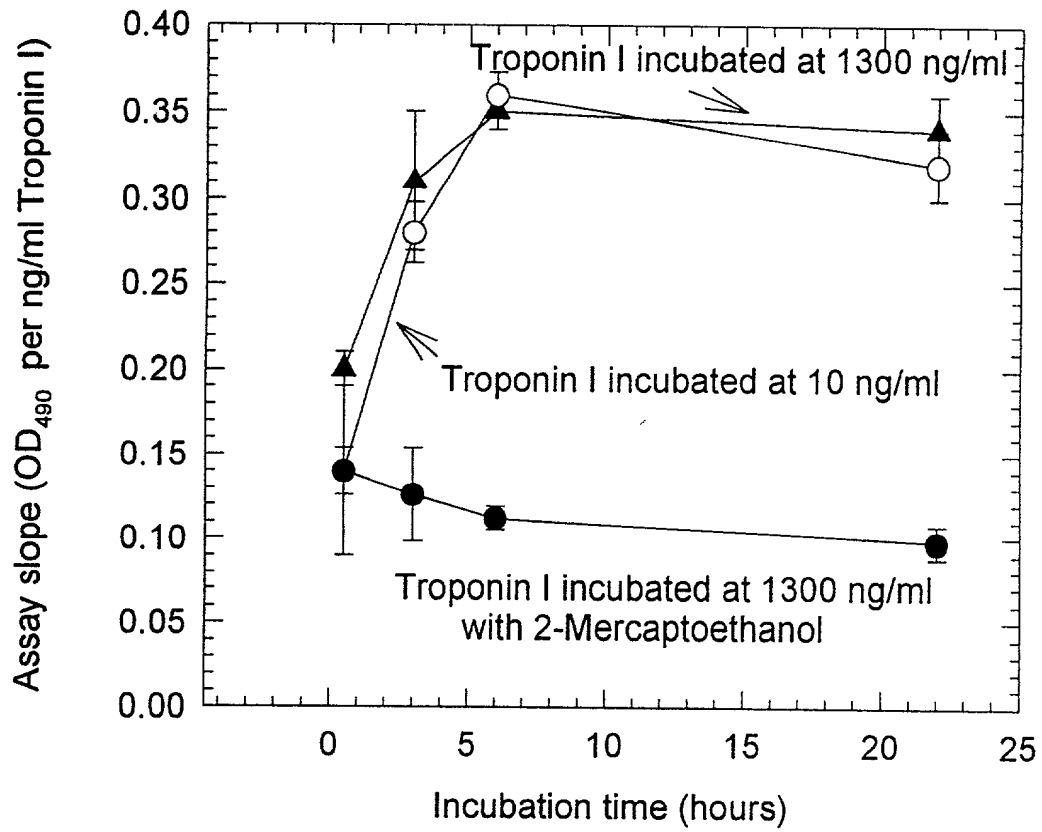


Figure 1b

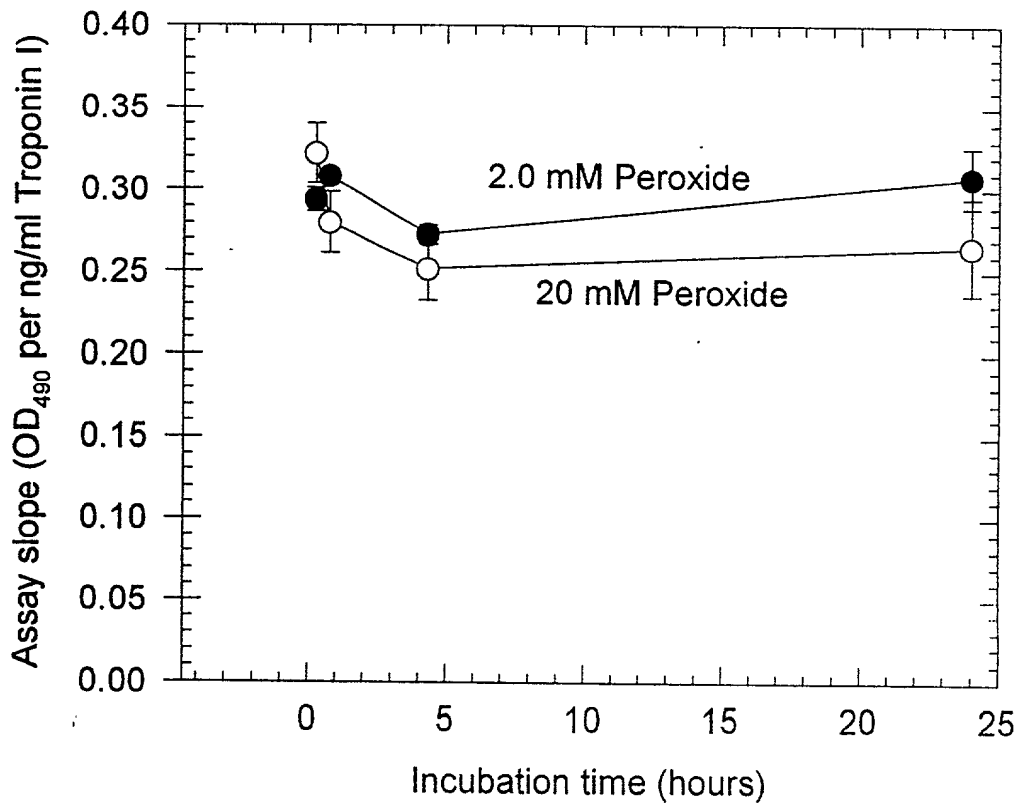


Figure 2

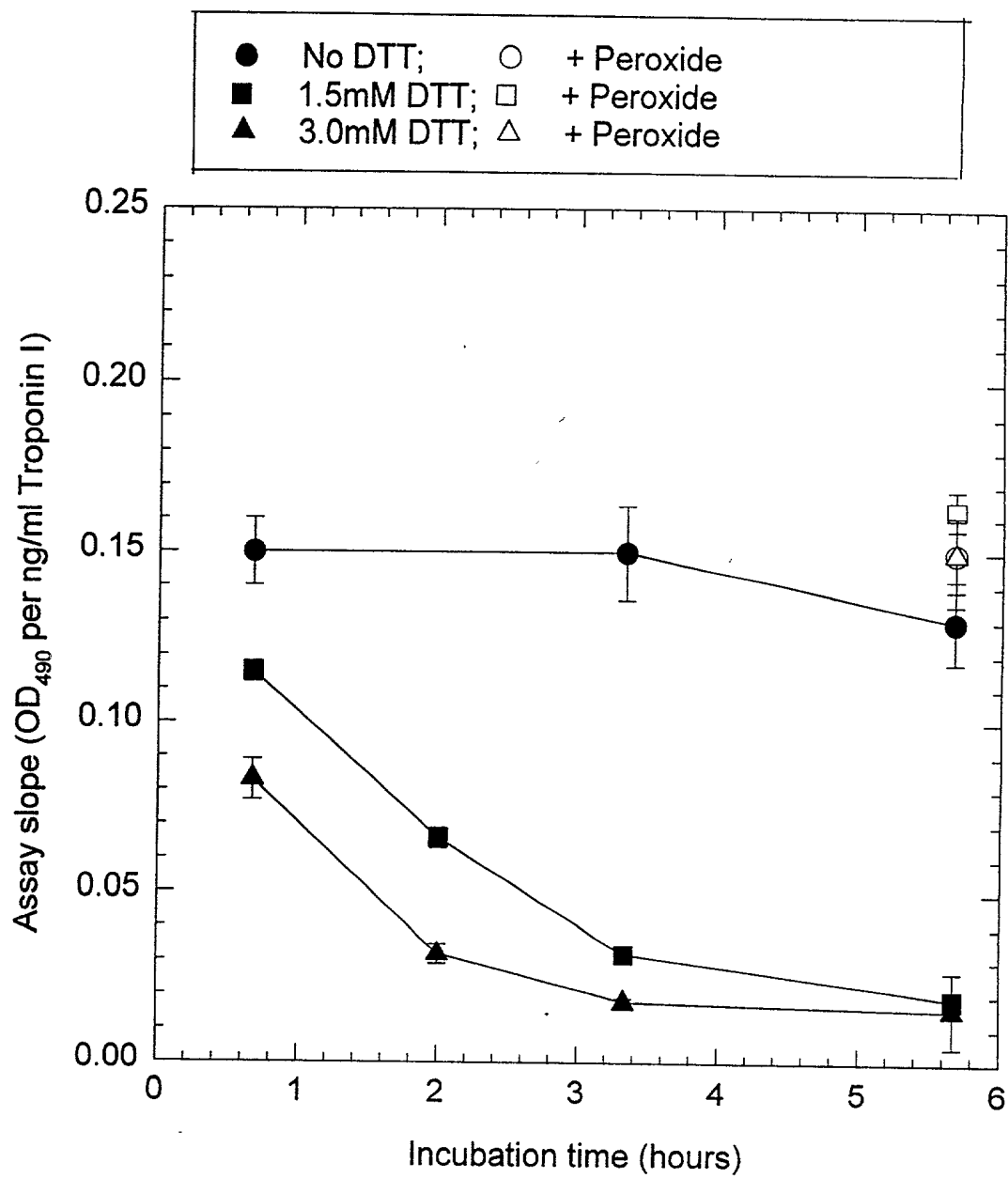


Figure 3

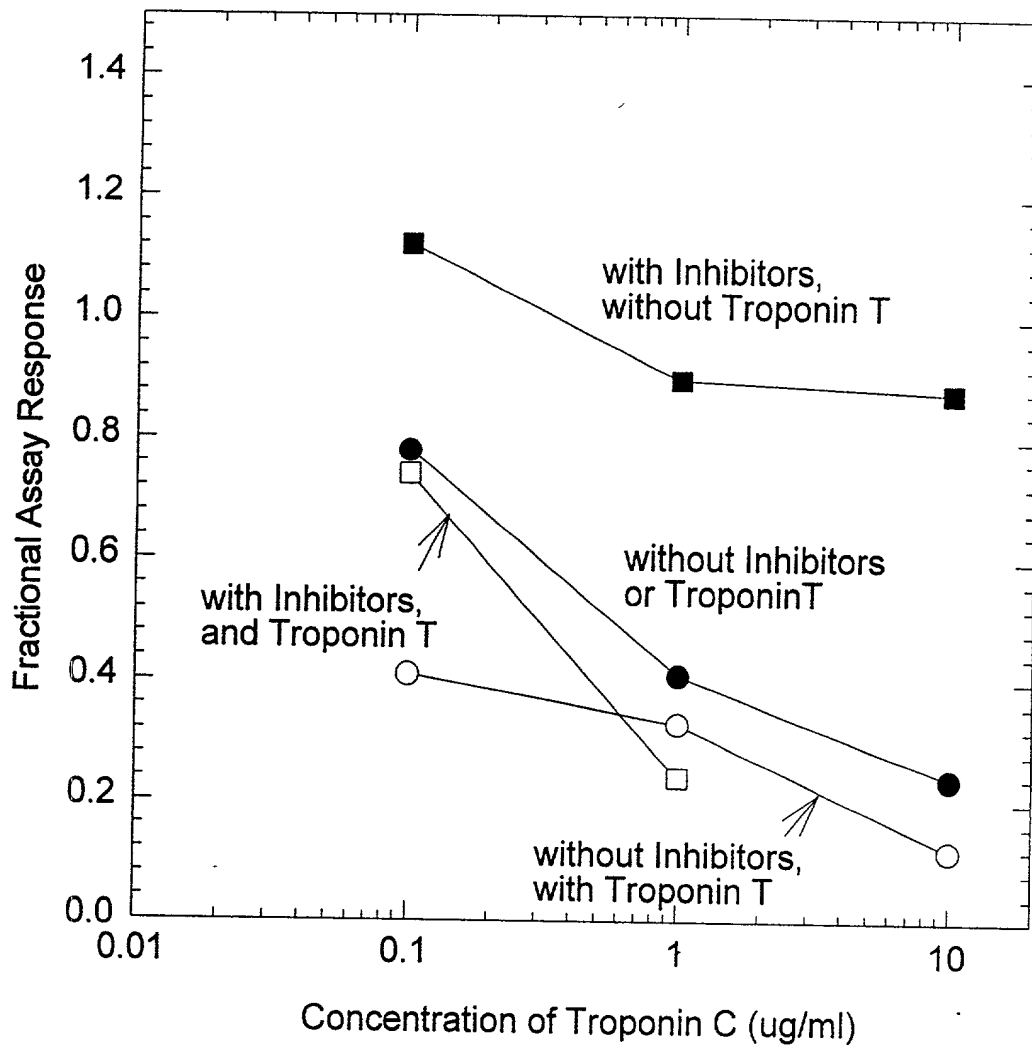


Figure 4

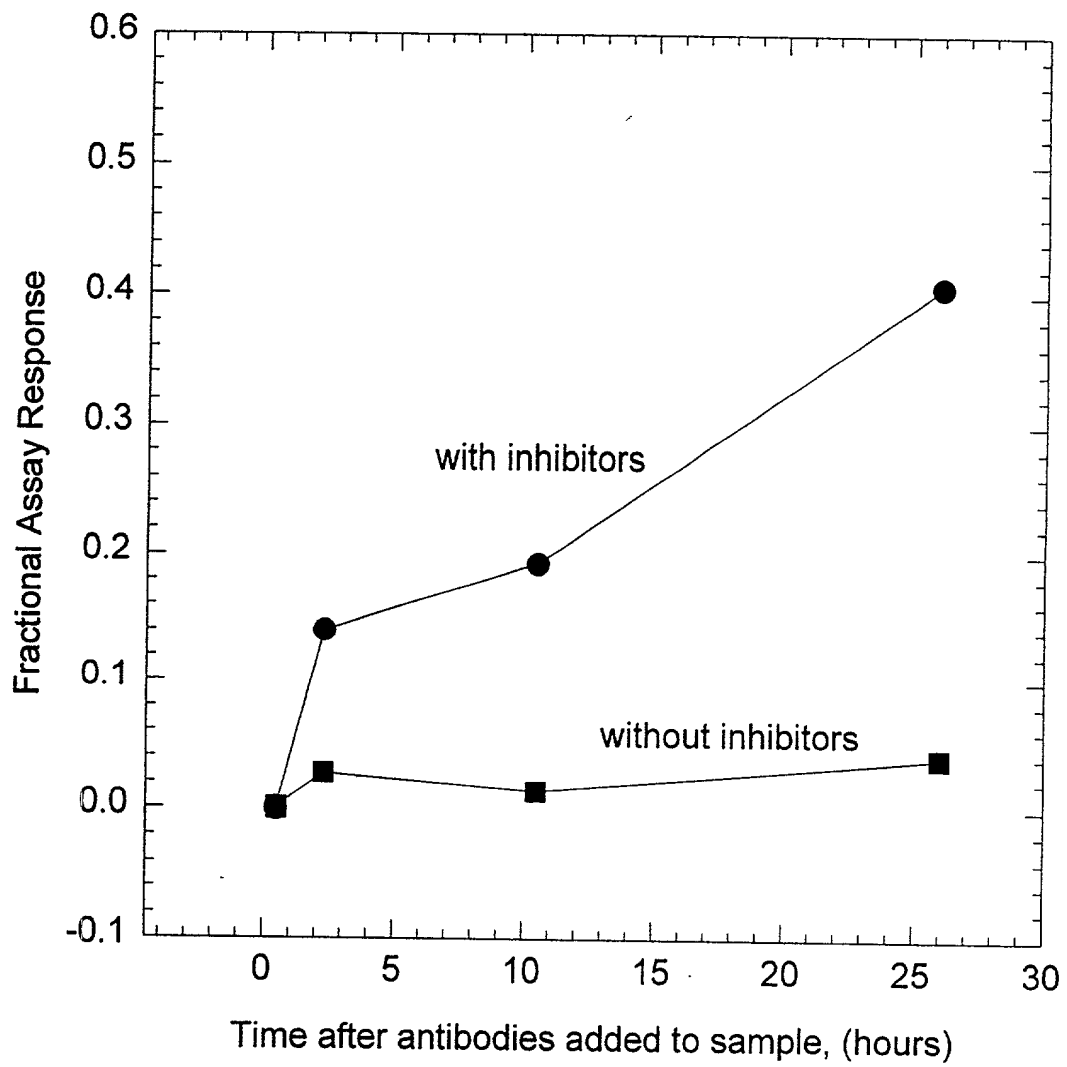


Figure 5a

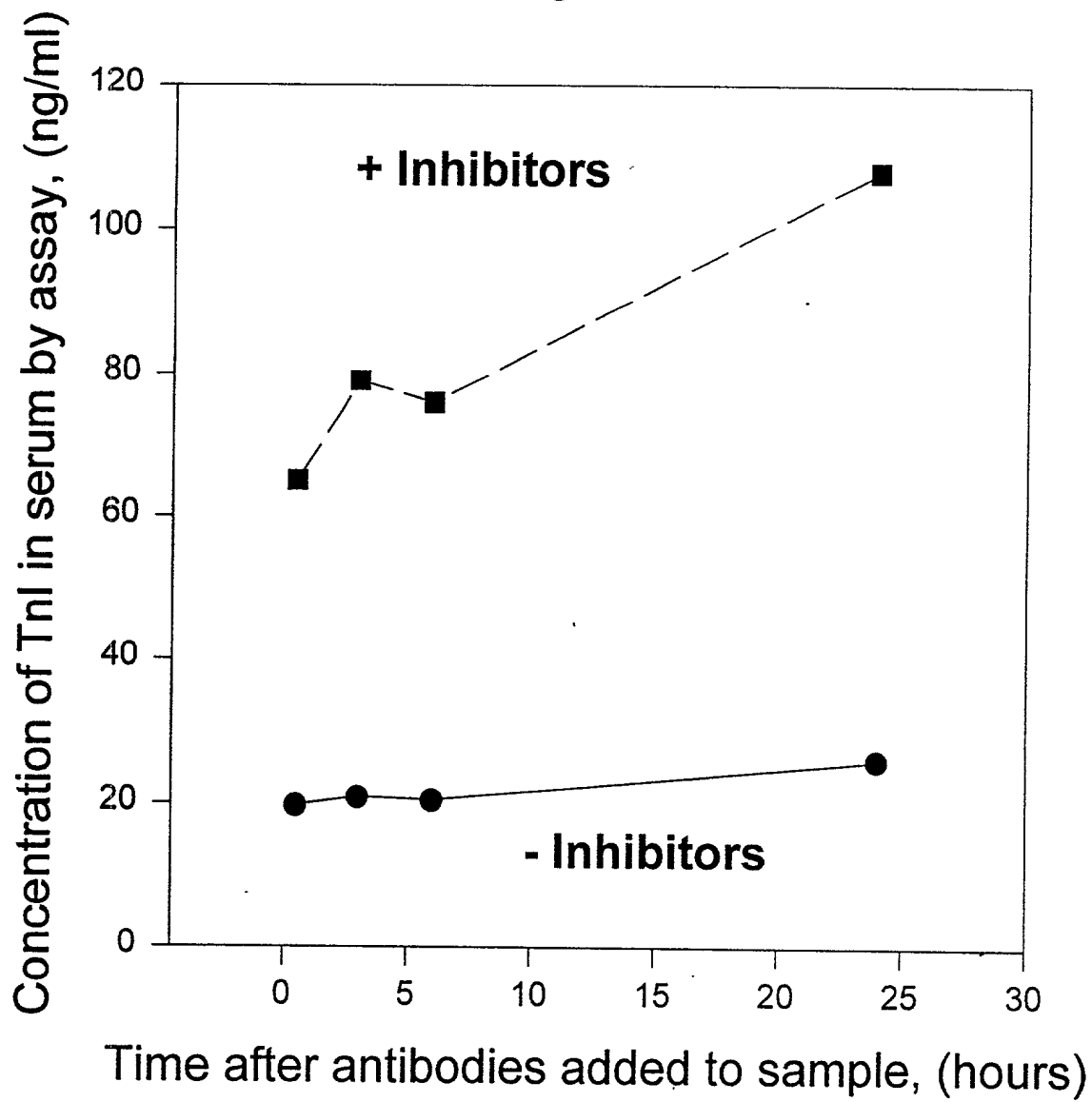


Figure 5b

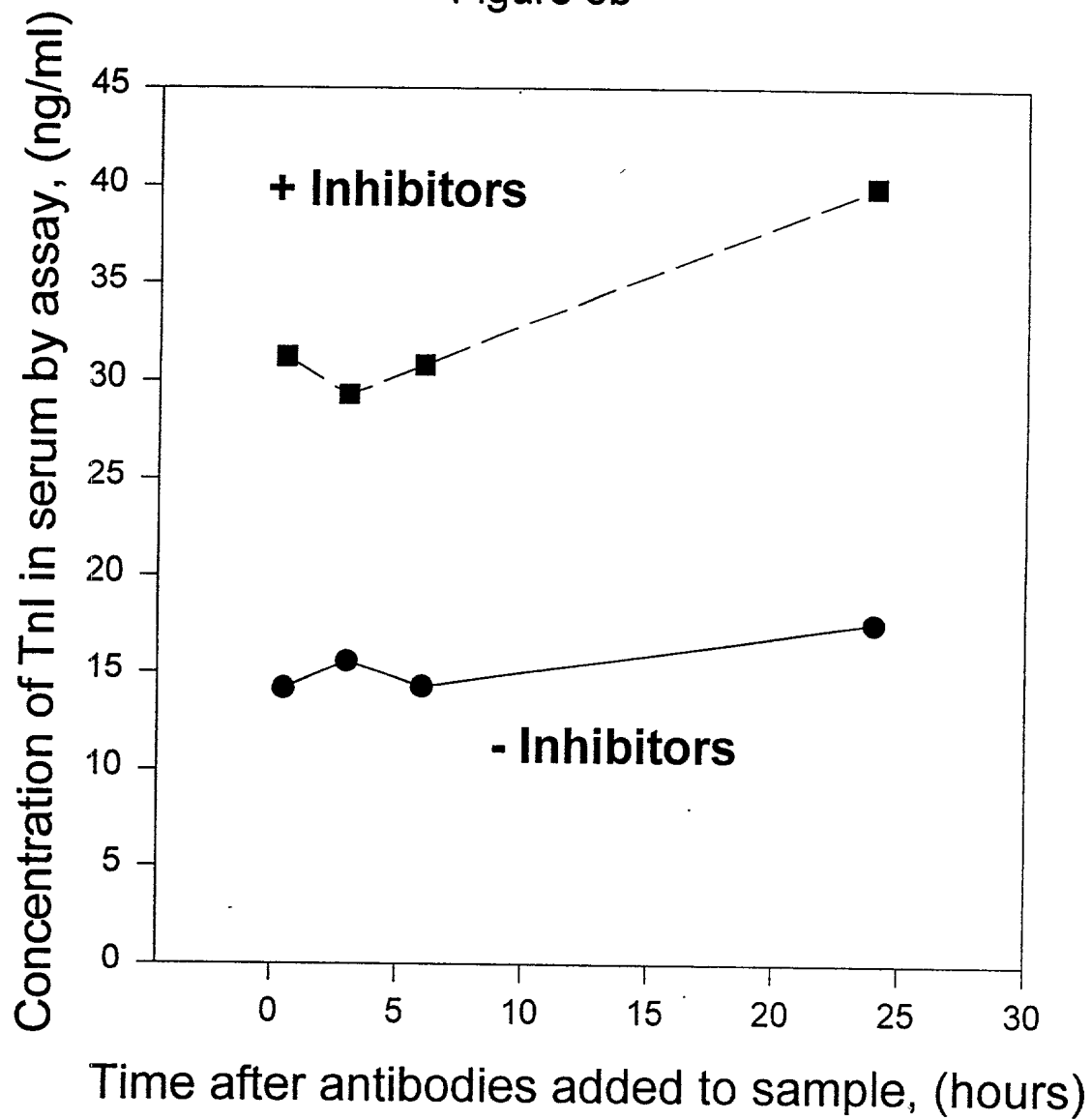




Figure 5c

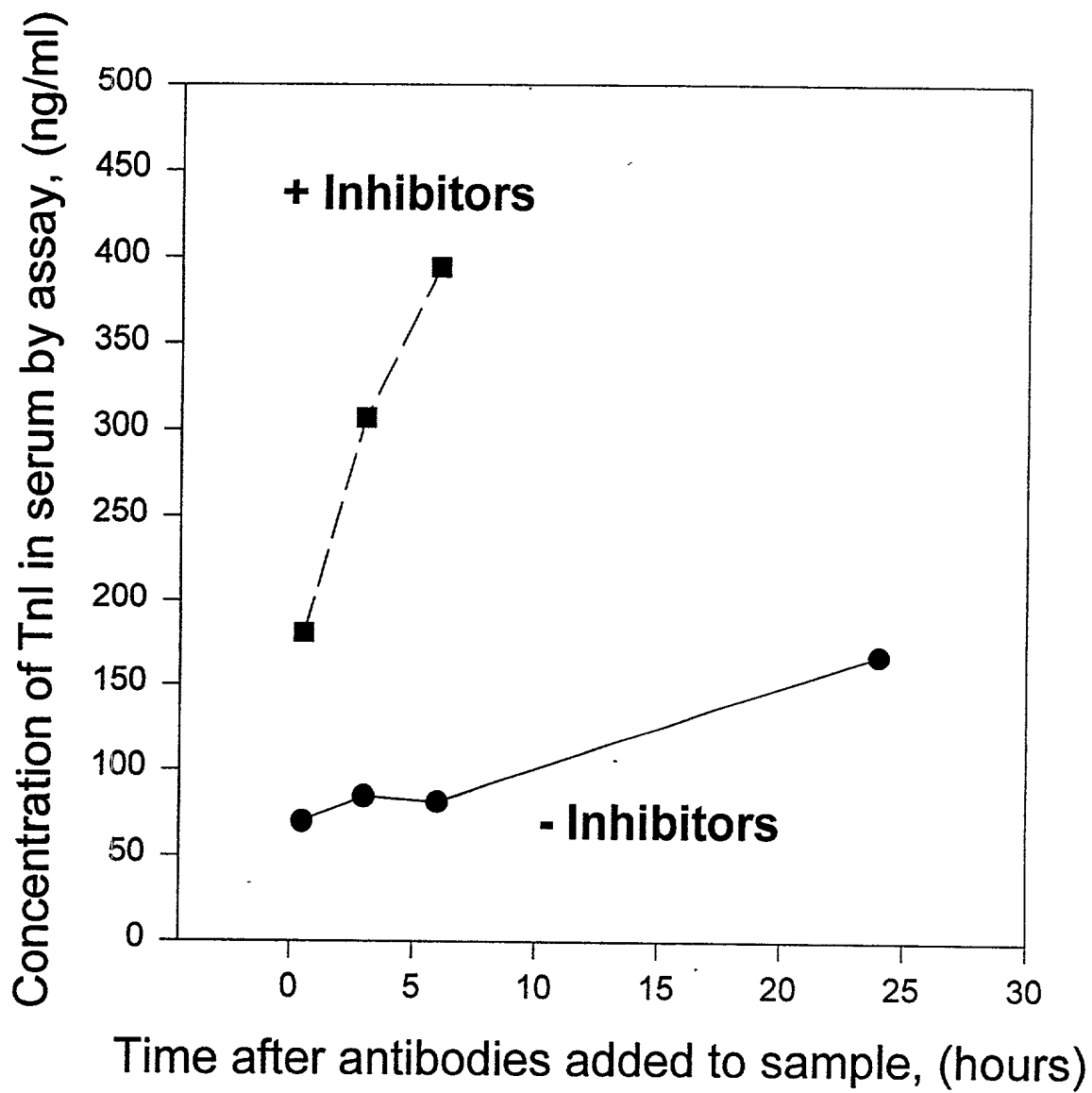


Figure 5d

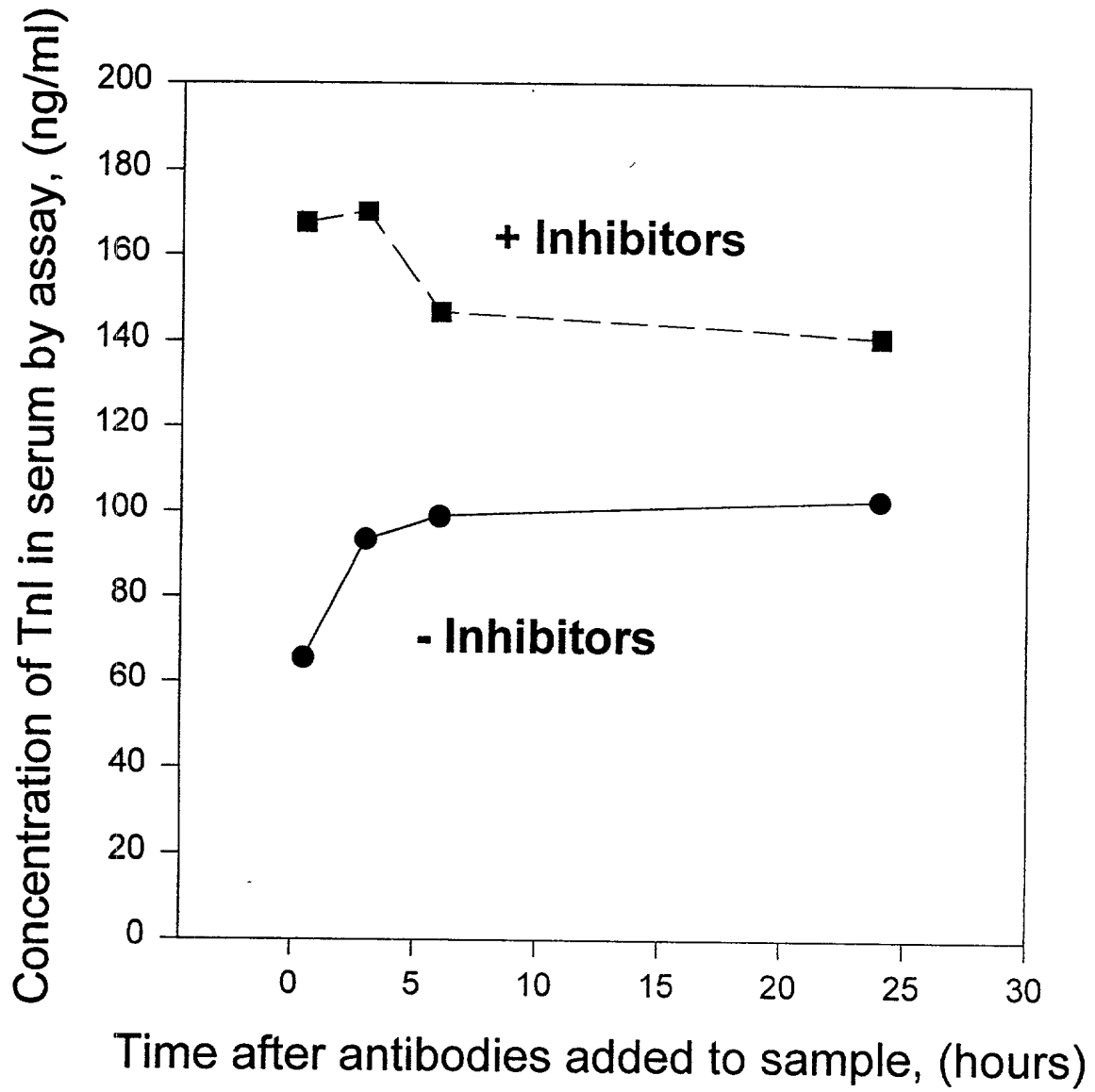


Figure 5e

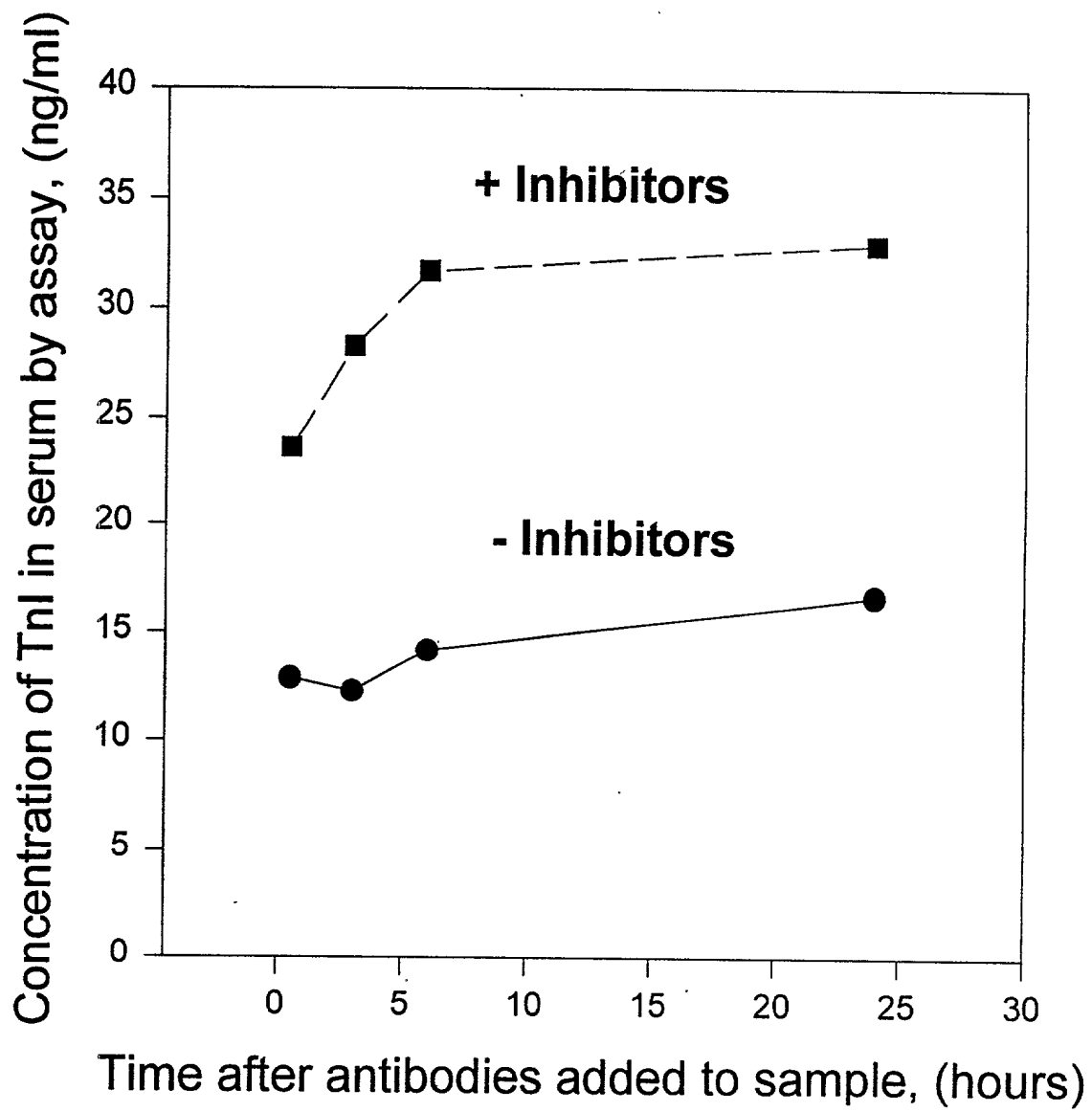
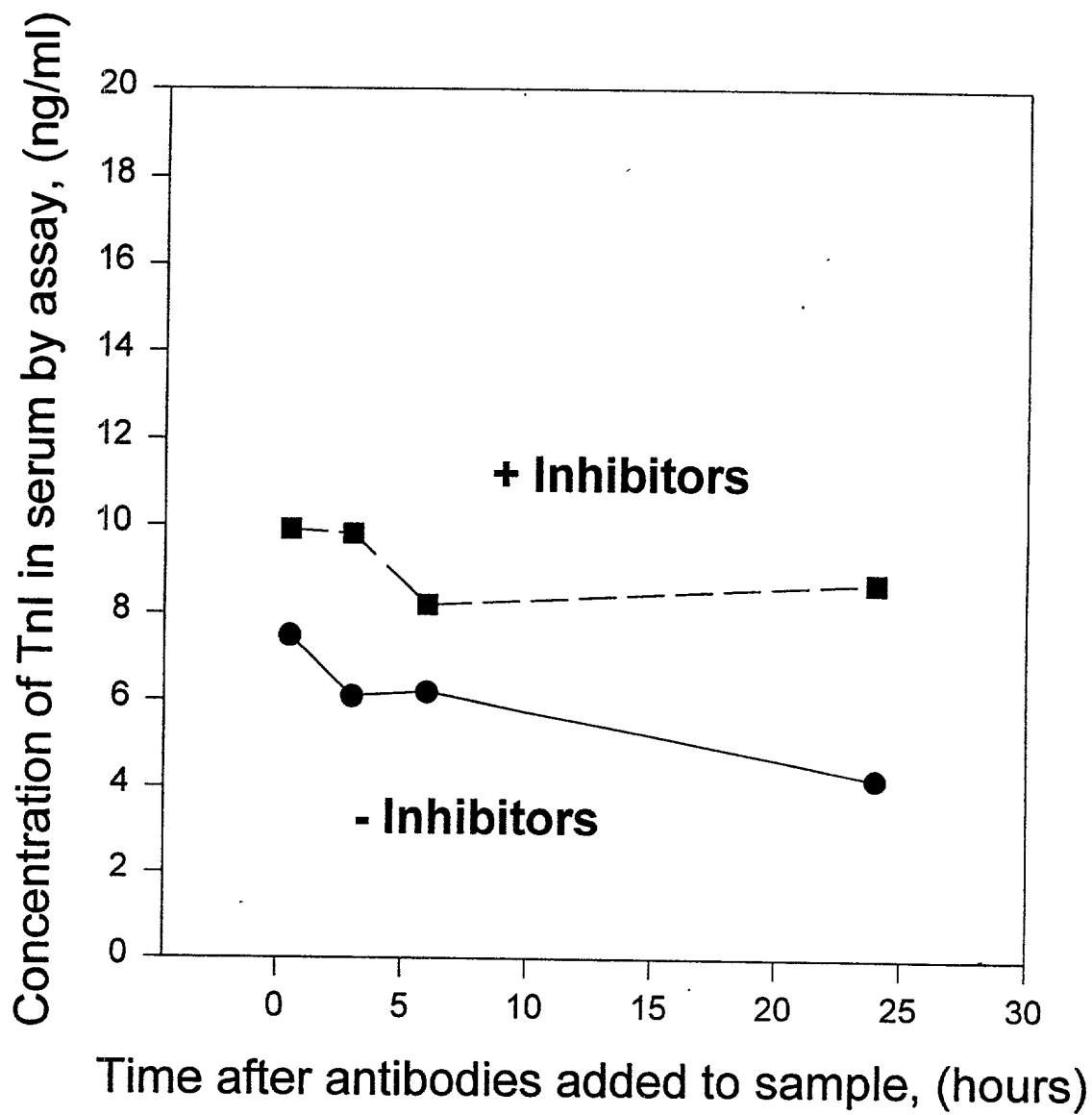


Figure 5f



# COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled  
NOVEL METHODS FOR THE ASSAY OF TROPONIN I AND T AND COMPLEXES OF TROPONIN I AND T AND  
SELECTION OF ANTIBODIES FOR USE IN IMMUNOASSAYS specification of which

\_\_\_\_\_ is attached hereto.

X was filed on 4/18/96 as Application Serial No. 08/633,248 and was amended on \_\_\_\_\_.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s):

(Number)	(Country)	(Day/Month/Year Filed)	Yes	No

(Number)	(Country)	(Day/Month/Year Filed)	Yes	No

(Number)	(Country)	(Day/Month/Year Filed)	Yes	No

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Timothy J. Lithgow, Reg. No. 36,856

Kindly recognize as associate attorney:

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Address all telephone calls to Timothy J. Lithgow at telephone no. 619-552-8400.

Address all correspondence to First Interstate World Center, 633 West Fifth Street, Suite 4700, Los Angeles, California 90071-2066.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issuing thereon.

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Citizenship United States of America

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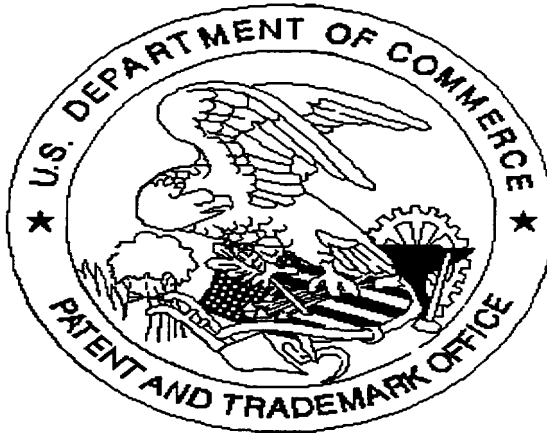
Inventor's signature  Date: 6/28/96

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Citizenship United States of America

Post Office Address \_\_\_\_\_

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